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Faculty of Veterinary Medicine, Leipzig University

Hair cortisol concentration in cattle and pigs: Investigation of influencing factors and the potential as an indicator of long-term stress

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Susen Heimbürge

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Dekan: Prof. Dr. Dr. Thomas Vahlenkamp

Betreuer: Prof. Dr. Uwe Truyen

Prof. Dr. Birger Puppe

Gutachter: Prof. Dr. Uwe Truyen, Institut für Tierhygiene und Öffentliches Veterinärwesen, Veterinärmedizinische Fakultät der Universität Leipzig, Leipzig

Prof. Dr. Birger Puppe, Institut für Verhaltensphysiologie, Leibniz-Institut für Nutztierbiologie (FBN), Dummerstorf

Prof. Dr. Dr. Michael Erhard, Veterinärwissenschaftliches Department der Ludwig-Maximilians-Universität München, München

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List of abbreviations

a.m.	*ante meridiem*
ACTH	adrenocorticotrophic hormone
ANOVA	analysis of variance
AUC	area under the curve
AVP	arginine vasopressin
BLE	Bundesanstalt für Landwirtschaft und Ernährung
BMEL	Bundesministerium für Ernährung und Landwirtschaft
CRH	corticotrophin-releasing hormone
CV	coefficient of variation
DNA	deoxyribonucleic acid
e.g.	*exempli gratia*
ELISA	enzyme-linked immunosorbent assay
Fig.	figure
g	gravitational field strength
GC(s)	glucocorticoid(s)
GL	German Landrace
GLW	German Large White
GR(s)	glucocorticoid receptor(s)
HCC(s)	hair cortisol concentration(s)
HPA	hypothalamic-pituitary-adrenal
HPLC	high-performance liquid chromatography
i.m.	intramuscular
IU	international units
LSM(s)	least squares mean(s)
MR(s)	mineralocorticoid receptor(s)
p	probability
p.m.	*post meridiem*
QS	Qualitätssicherung
r	correlation coefficient
rpm	revolutions per minute
RT	room temperature
SAS	statistical analysis system
SD	standard deviation
SE	standard error

S_H	saliva with high cortisol concentration
S_L	saliva low high cortisol concentration
U_H	urine with high cortisol concentration
U_L	urine low high cortisol concentration
UV	ultraviolet

1 General introduction

As a main system of stress regulation, the hypothalamic-pituitary-adrenal (HPA) axis induces the production and release of cortisol from the adrenal glands (SAPOLSKY et al. 2000, JACOBSON 2005). While short-term activation of the HPA axis is an adaptive physiological response to stressors and essential for survival, long-term increased cortisol release can harm an organism (MOBERG 2000, MCEWEN 2008). Farm animals, in particular, can be exposed to many different stresses and strains due to their husbandry conditions (WOOD-GUSH et al. 1975, FRASER et al. 2001). Previous studies have shown that stress in animals can lead, for example, to impaired muscle growth, reduced reproductive success, and immunodeficiency (BARNETT and HEMSWORTH 1990, TURNER et al. 2012). These consequences may lead to maladaptation and increased biological costs (distress), which can impair animal welfare, a complex concept that considers both physical and mental health and in which stress monitoring is an important element (TILBROOK and RALPH 2017). Since stress leads to an increase in cortisol secretion, this elevation in systemic cortisol concentrations is used as a biological marker in stress research (SPENCER and DEAK 2017). Usually, cortisol analysis is carried out in blood, saliva, urine, and faeces samples (MÖSTL and PALME 2002). These conventional biological matrices provide insights into cortisol concentrations from minutes to hours before sampling (COOK 2012). However, cortisol is released in a pulsatile, and circadian rhythm (JACOBSON 2005), so repeated measurements may be necessary to use these materials to assess long-term stress, that is, stress that occurs over several weeks to months (DHABHAR 2018). With a growing interest in studying long-term stress in farm animals, minimally or non-invasive methods are increasingly the focus of stress research and animal-welfare science.

About 20 years ago, scientists took up the results of forensic and drug research in human hair and analysed cortisol in this novel sampling material for the first time (CIRIMELE et al. 2000, KOREN et al. 2002). Cortisol is predominantly incorporated into a growing hair by diffusion from blood vessels (MEYER and NOVAK 2012), so hair provides average cortisol concentrations from the period of hair growth (weeks to years) (STALDER and KIRSCHBAUM 2012). Hair sampling is also minimally invasive, hair can be stored easily, and longer hair strands offer the possibility of creating a retrospective calendar (KIRSCHBAUM et al. 2009, RUSSELL et al. 2012). These numerous advantages of hair have stimulated research on hair cortisol analysis, not only in humans but increasingly in wild and domesticated animals as well (RUSSELL et al. 2012, BURNARD et al. 2017, MESARCOVA et al. 2017). Previous studies have investigated various determinants of variations in hair cortisol concentrations (HCCs) and have shown the dependence of HCC not only on stress-related factors but also on individual and environmental determinants (BURNARD et al. 2017, STALDER et al. 2017). However, basic knowledge about this relatively new method of cortisol assessment is still missing, especially in farm animals. With my current research, I would like to

contribute to compensating for this lack of knowledge by examining HCCs in cattle and pigs. Therefore, the general aims of the present work are to investigate influencing factors on HCC, including animal-based, seasonal and hair-specific factors as well as contamination and elimination, and to examine the potential of hair cortisol concentration as an indicator of long-term stress in cattle and pigs. The following chapters provide the most important background information for contemporary hair cortisol studies. After giving an overview of the principles of farm animal welfare and an explanation of the stress response in mammals, basic knowledge about hair as biological material is presented. In this context, different pathways of cortisol incorporation and elimination are described. Following this introductory part of the thesis, I give an overview of the specific aims and hypotheses of the present studies, which are presented in detail in the results section as publications comprising a literature review (Study 1) and three experimental studies (Studies 2–4). The present work is therefore a cumulative dissertation containing four peer-reviewed papers published in an international journal. Finally, all results are discussed in general against the background of the current state of knowledge, including an evaluation of the usability of hair cortisol concentrations in cattle and pigs and the presentation of future perspectives for further research and applications.

2 Review of the literature

2.1 The relevance of stress assessment in animal welfare

A common biological definition of stress introduced by BROOM and JOHNSON (1993) is 'an environmental effect on an individual which overtaxes its control systems and reduces its fitness'. SELYE (1975) differentiated the terms 'distress', the negative, pathological form of stress, and 'eustress', the positive form of stress with beneficial outcomes. The terms 'stress' and 'distress' are often used interchangeably, as negative stress is the most commonly mentioned type of stress. Thus, stress is a state of threatened physiological and/or psychological homeostasis to which the organism reacts with behavioural and physiological responses called 'adaptation' and 'coping' (LAZARUS and FOLKMAN 1984, SCHNEIDERMANN et al. 2005, CHROUSOS 2009). These stress responses are beneficial for the organism as long as they enable it to adapt to and overcome challenges. However, due to a severe stressful event or particularly long-term stress, coping and adaptation may fail to return an organism to physiological and/or psychological homeostasis, leading to maladaptation with deleterious effects on health and welfare (MOBERG 2000, YARIBEYGI et al. 2017).

The current understanding of animal welfare as a measure of the animals' quality of life has developed in the context of the social and cultural history of animal care and use and has roots in an expanding knowledge base of animal physiology and ethology (NATIONAL RESEARCH COUNCIL 2008). Broad public interest in animal welfare was first awakened by the publication of the book *Animal Machines* by HARRISON (1964), which depicted the husbandry conditions of the time and the associated suffering of farm animals (FRASER 2008). In reaction, the British government appointed a committee under the leadership of Professor F. W. Rogers Brambell to investigate 'the welfare of animals kept under intensive livestock husbandry systems' (BRAMBELL et al. 1965). A main finding of these investigations was that animal welfare is particularly dependent on both the physical and the mental wellbeing of the animal. To properly consider both these aspects and to grant animals basic freedoms, the Farm Animal Welfare Council developed 'five freedoms' based on the recommendations of the Brambell Report (WEBSTER 1995): 1) freedom from hunger and thirst, 2) freedom from discomfort, 3) freedom from pain, injury or disease, 4) freedom to express normal behaviour, and 5) freedom from fear and distress. Although the formulation of these freedoms was a good start towards improving conditions in animal husbandry, they reflect an ethical, anthropocentric view rather than a scientific one (KORTE et al. 2007). Another approach to defining animal welfare was therefore to examine both brain and periphery states in relation to the environmental challenges that led to those states (KORTE et al. 2007). Not constancy or freedoms, but capacity to change is crucial to good health and welfare (KORTE et al. 2007). In contrast to homeostasis, allostasis concept describes the process of achieving 'stability through changes'

(STERLING and EYER 1988) that enable an animal to respond to changing circumstances (MCEWEN and WINGFIELD 2010). BROOM (1986) formulated a widely used definition of welfare:

> 'The welfare of an individual is its state as regards its attempts to cope with its environment. Coping can sometimes be achieved with little effort and expenditure of resources, in which case the individual's welfare is satisfactory. Or it may fail to cope at all, in which case its welfare is obviously poor'.

How well an animal can cope with challenging situations depends on individual constitution, personality, previous experiences, and the nature of the challenging circumstances (KOOLHAAS et al. 1999, TILBROOK and RALPH 2017). Coping can be either neuroendocrine, behavioural, autonomic, immunological, or most of all a combination of these mechanisms (KOOLHAAS et al. 1999). Thus, animal-based indicators for the assessment of welfare comprise physiological measures (e.g. indicators of stress) as well as behavioural measures and clinical signs.

2.2 Stress response in mammals

2.2.1 Structure and function of the hypothalamic-pituitary-adrenal axis

A major system of the physiological stress reaction that plays an important role in the adaptive response to long-term stress is the hypothalamic-pituitary-adrenal axis (Fig. 1) (JACOBSON 2005).

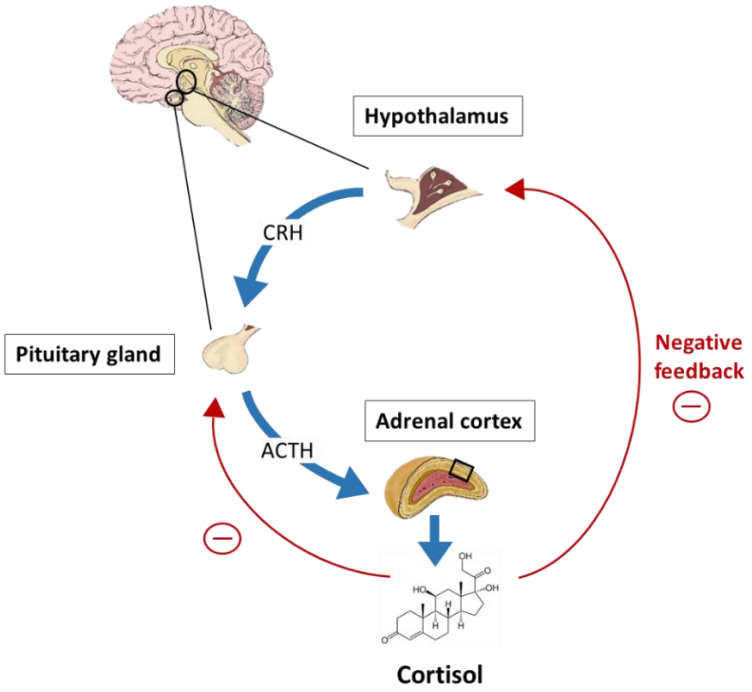

Fig. 1: Structure of the HPA axis; the response to stress (blue arrows) and the negative feedback loop (red arrows)

Neurons whose cell bodies are located in the hypothalamus (specifically, the *Nucleus paraventricularis*) produce corticotrophin-releasing hormone (CRH) and neurohormone arginine vasopressin (AVP) (SAPOLSKY et al. 2000, SPENCER and DEAK 2017). When an organism is confronted with stressors, CRH is released and stimulates the G-protein-coupled CRH-receptor-1 in the endocrine cells of the anterior pituitary (JACOBSON 2005). There, these corticotrophs release the adrenocorticotrophic hormone (ACTH), which is a cleavage product of proopiomelanocortin (SPENCER and DEAK 2017). Especially during repeated or chronic stimulation of the hypothalamus, AVP may function as a co-factor for the activation of ACTH-producing corticotrophs (SPENCER and DEAK 2017). In the adrenal glands (specifically, the *zona fasciculate*), ACTH leads to the conversion of cholesterol into glucocorticoids (GCs) by stimulating the melanocortin-2 receptor and finally the release of GCs into the blood (JACOBSON 2005). Hence, the HPA axis is a classical neuroendocrine system that serves to control adrenocortical GC secretion by the brain. Glucocorticoids, such as cortisol and corticosterone, have numerous effects on peripheral tissues, which can roughly be summarised as the organisation of energy consumption and energy distribution to overcome the homeostatic challenge caused by stress (JACOBSON 2005). Furthermore, the increased secretion of GCs has a regulating effect on the HPA axis itself. As a consequence of this negative feedback, the release of CRH and ACTH is inhibited, thus reducing the production of GCs. Therefore, the plasma levels of GCs are always within a species-specific physiological range if all organs involved are functioning properly.

2.2.2 Characteristics and effects of cortisol

The main glucocorticoid in most mammals and fishes is cortisol, whereas in rodents and birds it is corticosterone (JACOBSON 2005, SPENCER and DEAK 2017). Cortisol belongs to the class of steroid hormones and is released in a pronounced pulsatile and circadian rhythm depending on the day-night cycle. In diurnally active animals, cortisol peak levels are observed during the early morning, while nadir levels occur in the evening. In nocturnal animals, this cycle is reversed (JACOBSON 2005, FRIES et al. 2009, MÖSTL 2014). Steroid hormones are characterised by their typical biochemical structure of 17 carbon atoms arranged in four ring systems (GRANNER et al. 2015). Due to its liposolubility, the cortisol molecule can passively diffuse into cells across the phospholipid bilayers and activate intracellular receptors. There are two GC receptors: the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR) which both belong to the transcription factor (REUL and DE KLOET 1985, GRANNER et al. 2015). After binding cortisol in the cytoplasm, the receptor is activated and translocated into the cell nucleus. Locally, the receptor binds to deoxyribonucleic acid (DNA), modulates the transcription of specific genes, and thereby influences the synthesis of proteins (DE KLOET et al. 1998, 2005). Besides this receptor-mediated regulation of gene expression (genomic effects), which reacts with a slight delay, steroid receptors

also act due to rapid, receptor-mediated effects (non-genomic effects) (GROENEWEG 2011). This DNA-binding-independent functioning can be achieved due to protein-protein interactions with other transcription factors and enables, for instance, the negative feedback of cortisol on the HPA axis (GROENEWEG 2011). Cortisol can react with both MRs and GRs but binds with a tenfold higher affinity to MRs (SPENCER and DEAK 2017). Due to its numerous biological functions, cortisol is not only an important hormone in the stress response but also has various regulatory effects in the whole organism. As part of the provision of energy substrates, cortisol inhibits the uptake of glucose into cells and stimulates gluconeogenesis, which increases the glucose level in the bloodstream (KUO et al., 2015). Additionally, it inhibits lipogenesis, increases the decomposition of proteins and bones, and thereby influences muscle growth and the occurrence of osteoporosis (LUKERT and RAISZ 1990, GRANNER et al. 2015). In the gastro-intestinal system, the blood supply and secretion of digestive enzymes, protective mucus and saliva declines, while the production of gastric acid increases, elevating the risk of ulcer formation due to excessive cortisol release (YARIBEYGI et al. 2017). In addition, cortisol can increase blood pressure and heart rate and inhibit the immune system through various mechanisms (GRANNER et al. 2015, YARIBEYGI et al. 2017). Furthermore, cortisol facilitates the foetal development of organs such as the eyes, nervous system, and lungs (CHALLIS et al. 2001, GRANNER et al. 2015).

2.2.3 Conventional biological matrices for cortisol analysis

Cortisol is commonly used as an individual-based physiological indicator to assess stress, and its altered secretion is traditionally measured in blood samples (MORMÈDE et al. 2007). In mammals, cortisol is transported in the bloodstream mainly by binding to proteins, such as corticosteroid-binding globulin. About 90% of systemic cortisol is bound, while the remaining 10% is unbound in the bloodstream (MENDEL 1989, SPENCER and DEAK 2017). According to the free hormone transport hypothesis (MENDEL 1989), only unbound cortisol can diffuse into target tissues, so the cortisol concentrations detected in saliva samples, for example, are a reflection of this free cortisol fraction. Due to its simple and minimally invasive sampling procedure, salivary cortisol is applied increasingly frequently, especially for the repeated determination of cortisol in human and animal research (NEGRÃO et al. 2004, HELLHAMMER et al. 2009, MUNSTERHJELM et al. 2013). After a short half-life of about 15 minutes, cortisol is converted into biologically inactive, water-soluble metabolites that are predominantly excreted with urine and to a lesser extent with faeces (MÖSTL and PALME 2002, SPENCER and DEAK 2017). Cortisol and its metabolites can therefore also be determined in urine and faeces samples. All these biological matrices have their advantages and disadvantages, which various review articles have presented in detail (MORMÈDE et al. 2007, NOVAK et al. 2013, SPENCER and DEAK 2017). Blood, saliva, urine and faeces samples differ, for example, in their stability and storage possibilities, invasiveness of collection and

dependence on the circadian rhythm. Cortisol measurements in blood and saliva samples reflect only the short-term hormone levels of the preceding minutes or hour and are therefore best suited to assess acute stress. Cortisol analysis in urine and faeces reveals average cortisol concentrations over periods ranging from hours (urine) to days (faeces), so if the aforementioned conventional matrices are used to assess long-term stress (weeks to month), it would be necessary to perform repeated sampling. Furthermore, the cortisol concentration in these biological materials is strongly influenced by short-term fluctuations in cortisol release, such as circadian rhythms, stress due to the sampling procedure, food intake, and exercise (OTOVIC and HUTCHINSON 2015).

2.3 Hair as a matrix for cortisol analysis

2.3.1 Hair structure and hair types

In mammals, hair is a skin appendage located in the epidermis and surrounded by sweat glands, sebaceous glands, and the erector muscle (HARKEY 1993, PRAGST and BALIKOVA 2006). Macroscopically, hair can be categorised into two main parts: the hair shaft and hair root (BOUMBA et al. 2006) (Fig. 2). The hair shaft is a dead, metabolically inactive tissue (PRAGST and BALIKOVA 2006) that protrudes above the skin and is therefore visible. The hair shaft consists of protein complexes (65–95%), water (15–35%), lipids (1–9%), pigments, and small quantities of trace elements (KIDWELL and BLANK 1996, ROBBINS 2002), and it has three layers: the cuticle, cortex, and medulla (CRUZ et al. 2016). The cuticle is an outer hydrophobic protection layer. Interestingly, although the cuticle is hydrophobic, its inner layers have hydrophilic properties and are therefore prone to swelling in aqueous liquids (ROBBINS 2002, KIDWELL and SMITH 2007). As a result, hair samples that appear dry from the outside may contain up to 30% moisture by weight (ZAHN 1989, CRUZ et al. 2016). The cuticle consists of flat, keratinised cells that overlap like roof tiles (HARKEY 1993, KIDWELL and BLANK 1996). The largest part of the hair shaft is called the cortex and consists of long, stratified, keratinised cells that form spindle-shaped fibres (CRUZ et al. 2016). This typical structure of macro- and microfibrils connected by cell membrane complexes is the reason for the flexibility of the hair shaft (ROBBINS 2002). Additionally, the cortex contains pigment granules filled with melanin, which gives the hair a typical colour and supports its photoprotective properties by dissipating absorbed ultraviolet radiation (HOTING et al. 1995, MADEA 2004). Melanin is produced in melanocytes located in the distal part of the hair root. There are two types of melanin: eumelanin (brown and black) and pheomelanin (red) (BOUMBA et al. 2006, CRUZ et al. 2016). The central part of the hair shaft is the medulla. It is surrounded by the cortex and consists of a thin cord of larger cells among air-filled spaces. The function of the medulla is still not fully understood, and not all hairs contain this part (HARKEY 1993, ROBERTSON 1999).

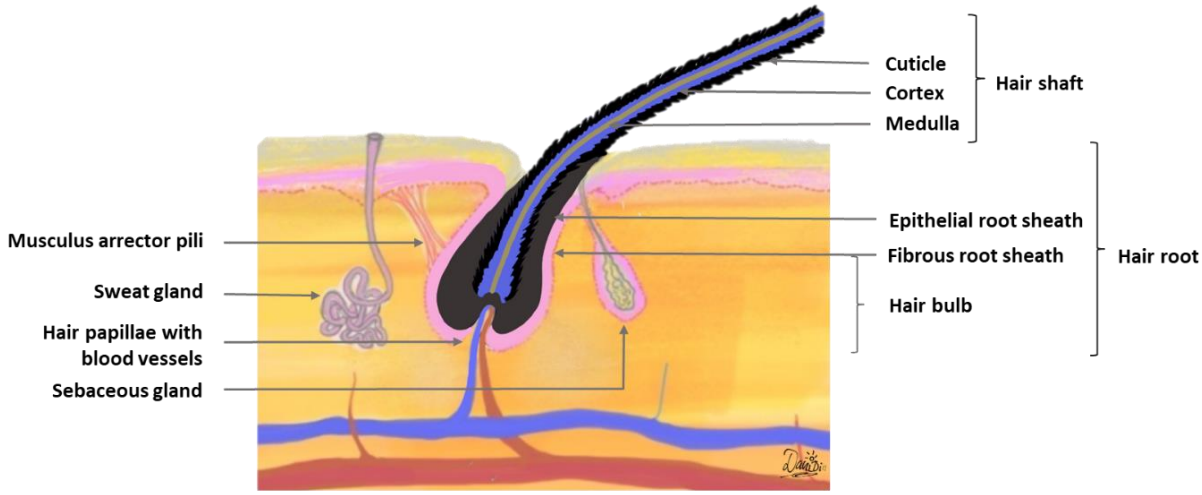

Fig. 2: Hair structure

The second main part of the hair – the hair root or hair follicle – is located approximately 2–4 mm below the skin surface (cattle: 2 mm (UDO 1978), pigs: 3.5 mm (personal data), humans: 4 mm (MADEA 2004)). The lowest part of the hair follicle is thickened and forms the hair bulb (CRUZ et al. 2016). An invagination at the basal end of the bulb forms the hair papilla, through which the hair is supplied with blood vessels. Besides the hair bulb, the hair follicle also consists of an outer and an inner epithelial root sheath and a fibrous root sheath (ROBERTSON 1999, CRUZ et al. 2016). The epithelial root sheath is an invagination of the epidermis, the hair shaft's anchor in the skin and the site of hair growth. The outer part of the hair root is the fibrous root sheath. This is where the musculus arrector pili is attached, which enables the hair to straighten up and contribute to thermoregulation (ROBERTSON 1999).

Due to the slightly inclined positions of hairs in the skin, they have specific growth directions which points in the direction of movement on the body and from proximal to distal on the legs (SALOMON et al. 2008). Hair density is species-specific and seasonal, and it depends on the number of active hair follicles (MOWAFY and CASSENS 1976b). However, the total number of hair follicles is already determined at the time of birth (MOWAFY and CASSENS 1976b, CRUZ et al. 2016). Hair types can be differentiated by their size and structure (SALOMON et al. 2008). The dominant hair type in most mammals is guard hair, which forms the main part of the fur, causes species-specific coat patterns and serves as a protective insulation layer (KONDO 2001; SALOMON et al. 2008). Additionally, species such as bears, boars, dogs and sheep have wool hairs, which are thin and puckered hairs that form an undercoat for thermal insulation (MEYER and GÖRGEN 1986, KONDO 2001, MEINDERS 2017). Pigs are covered with special guard hairs called bristles. These are stiff hairs with splitting ends, which in other mammalian species only appear as protective hairs around the head, such as eyelashes (SALOMON et al. 2008, MOHAN et al. 2015). Particularly striking are long

hairs, which appear, for example, as manes and tails in horses or as tail tips in cows and are characterised by an exceptionally long growth phase (HARKEY 1993, SALOMON et al. 2008). Another special case among the hair types is tactile hairs, which are surrounded by a blood sinus and many nerve fibres (SARKO et al. 2011).

2.3.2 Hair growth cycle

Hair growth takes place in the hair bulb and occurs in cycles of active growth and resting phases (CHASE 1954), with the rate of hair growth depending on the species and body region (HARKEY 1993, MADEA 2004). Active growth only occurs in the anagen phase, which is the longest stage and lasts , for instance, about 3–5 months in pigs (MOWAFY and CASSENS 1976a) and about five years in human scalp hair (CRUZ et al. 2016). During this growth phase, hair follicle cells differentiate and enable the hair shaft to grow (HARKEY 1993, ROBERTSON 1999). Additionally, melanocytes are activated to produce pigments. Since the hair follicle is supplied with blood via the papillae in the anagen phase, the incorporation of substances derived from the blood mainly occurs during this period (MEYER and NOVAK 2012). The anagen is followed by the transitional phase, known as catagen, which lasts about 20 days in pigs (MOWAFY and CASSENS 1976a) and several weeks in humans (MADEA 2004). The blood vessels begin to recede, pigmentation and differentiation discontinue, and the hair bulb shortens (ROBERTSON 1999). Finally, the hair reaches the resting phase, which is called telogen. In human scalp hair, this phase lasts about three months (CRUZ et al., 2016), whereas in animals, the duration of this resting phase is strongly influenced by environmental conditions (LING 1970). In the telogen phase, the hair stops growing completely and is finally expelled by the appearance of a new hair (ROBERTSON 1999). In humans, scalp hairs grow in a mosaic pattern, so hairs are always in different growth stages beside each other. However, growth stages undergo seasonal changes, showing maximum telogen hairs in August and September (ROBBINS 2002). In most domestic mammals, hair loss is observed as a seasonal change of hair in the spring and autumn, a phenomenon called shedding (LING 1970, STENN and PAUS 2001). Hair growth occurs mainly in waves with the synchronised activity of hair follicles (LING 1970, MEYER et al. 1980). In this way, animal species can adapt to changing climatic conditions. In domesticated pigs, the growth wave starts in the abdominal area and moves caudally over the sides to the sacral part of the back. From here, it then spreads cranially over the flanks and back to the head (MEYER et al. 1980). Due to domestication, a clear distinction between spring and winter shedding is not possible. However, at the beginning of summer, follicle activity increases and the hair coat becomes denser (MOWAFY and CASSENS 1976a). In cattle, clear shedding can be observed in the spring, with the whole body covered with loose, dull hairs (MEYER et al. 1980). In European cattle breeds, new summer hair first appears on the head and neck and gradually spreads towards the shoulders (MEYER et al. 1980). The growth wave of the regionally, synchronously developing

hair follicles then follows a narrow strip caudally along the spine, from where it spreads to the lateral body wall. The ventral flanks and abdominal region are the last parts of the body affected by the hair change. In contrast, shedding in autumn is comparatively inconspicuous, does not follow any pattern and is rather diffuse (MEYER et al., 1980).

2.3.3 Incorporation of cortisol into the hair

2.3.3.1 Passive diffusion

The first evidence that hair can store substances permanently emerged in the mid-19th century when arsenic was detected in the hair of a corpse (MADEA 2004). Almost 150 years later, CIRIMELE et al. (2000) detected cortisol in human hair and KOREN et al. (2002) in the hair of wildlife. Because research on the incorporation of drugs into human hair is more advanced than investigations of hair cortisol, the hypotheses on the incorporation of cortisol into hair were mostly adopted from drug research. At the beginning of hair research, it was assumed that substances were only incorporated into hair by passive diffusion from blood vessels that enter the hair root via the hair papillae (HENDERSON 1993). Since steroids are lipophilic hormones and can penetrate biological membranes passively, diffusion from the bloodstream seems the most important way to incorporate cortisol into the hair (HENDERSON 1993, MEYER and NOVAK 2012). Therefore, unbound cortisol from the blood is incorporated into the hair shaft caused by the concentration gradient and thus may reflect systemic cortisol concentrations. However, there is no evidence of where exactly steroid hormones are stored within the hair shaft. Studies from drug research have suggested that the stable retention of substances is mainly due to their binding to melanin and proteins (CONE 1996). Incorporation via passive diffusion is dependent on the blood supply, so only actively growing hair during the anagen phase can incorporate cortisol from the bloodstream (MEYER and NOVAK 2012). In addition, there is a time delay between cortisol incorporation, which takes place several millimetres below the skin surface, and when this hair section emerges from the skin surface (RUSSELL et al. 2012).

2.3.3.2 Multi-compartment model

Besides the diffusion of cortisol from blood vessels into growing hair, substances can be incorporated into the hair shaft from other sources (Fig. 3). The multi-compartment model proposed by HENDERSON (1993) assumes that substances from the skin or the environment can also be integrated into the keratinised hair shaft (BOUMBA et al. 2006). Due to the structure of the hair and its cuticle, substances that are lipophilic, non-polar and have radii of less than 0.4 nm can easily penetrate the hair shaft (MADEA 2004). Previous studies showed that body fluids containing cortisol, such as sebum, sweat, saliva and urine, can be additional sources of cortisol (MACBETH et al. 2010, CATTET et al. 2014, GRASS 2017). Sebum is a complex mixture of lipids and cell debris

produced in small follicle-associated holocrine glands (sebaceous glands) and secreted directly into the hair canal (HARKEY 1993, ZOUBOULIS et al. 2016). It keeps the hair smooth and protects the skin from desiccation (ROBBINS 2002). Additionally, eccrine sweat glands produce and secrete sweat on the skin surface. Sweat is an aqueous solution consisting mainly of water and NaCl, and it is primarily involved in thermoregulation (BAKER 2019). Due to the proximity of the hair and these glands, cortisol-containing body fluids cover the hair surface and thus enable the incorporation of cortisol into the hair shaft. The hair, especially in animals, can also be contaminated with saliva and urine that contain cortisol (MÖSTL and PALME 2002). Like external contamination with body fluids, WANG et al. (2019) showed that exogenous cortisol from a cortisol-containing cream can be incorporated into the hair shaft. Furthermore, follicle cells themselves may produce cortisol and form an independent 'peripheral HPA axis' (ITO et al. 2005, SLOMINSKI et al., 2007). However, the quantities of this peripheral cortisol are comparatively small and therefore play only a subordinate role.

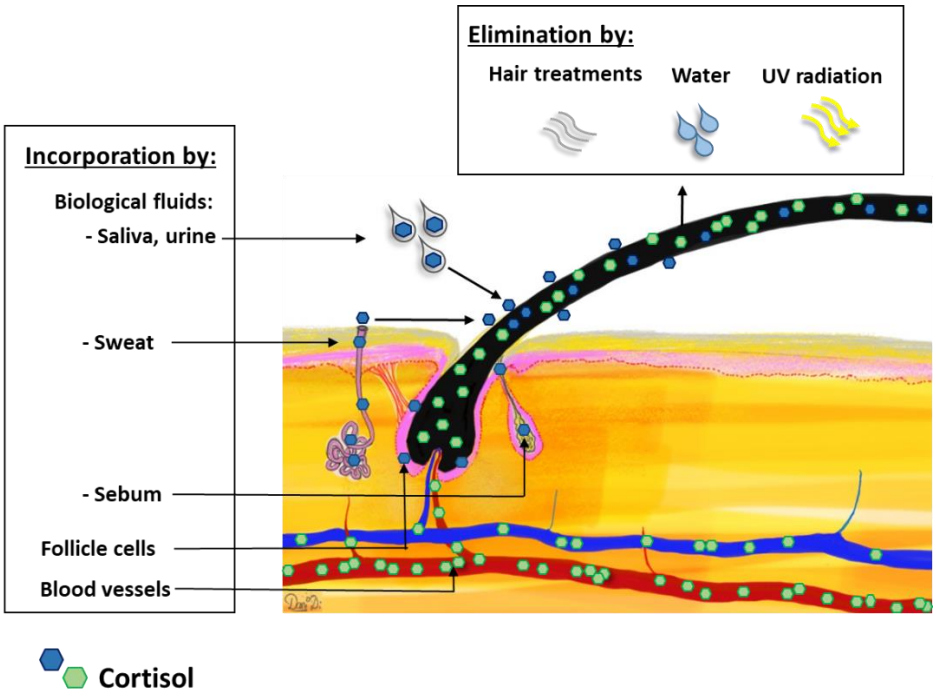

Fig. 3: Pathways of incorporation and elimination of cortisol into/from the hair shaft (adapted from MEYER and NOVAK 2012, STALDER and KIRSCHBAUM 2012, GRASS 2017). Blood cortisol, the presumed main source of incorporated cortisol, is marked by green hexagons (⬢) and cortisol from other sources by blue hexagons (⬢).

2.3.4 Elimination of cortisol from the hair

Previous studies on human hair have shown that extensive cosmetic treatments, such as dyeing and bleaching, can reduce concentrations of substances in the hair shaft (JURADO et al. 1997, BOUMBA et al. 2006). In this context, recent studies have investigated the impacts of different hair treatments on cortisol concentrations in hair and revealed contradictory results (HAMEL et al. 2011, DETTENBORN et al. 2012, KRISTENSEN et al. 2017). However, a meta-analysis of the various determinants of hair cortisol in humans showed that the influence of hair-washing frequency and hair treatment might be low (STALDER et al. 2017). Nevertheless, STALDER et al. (2017) recommended that the impact of these factors should be considered in future research. Frequent washing or chemical and physical treatment, such as dyeing, bleaching and brushing, may damage hair cuticles (DAWBER 1996, ROBBINS 2002, DIAS 2015) and thus leach cortisol. When examining hair segments by cutting hair strands into several pieces and analysing them separately, KIRSCHBAUM et al. (2009) observed a decline in cortisol concentrations from the proximal to the distal part of the hair, the so-called 'washout effect'. STALDER et al. (2017) confirmed these findings and showed a decline of 29% in HCC from the first proximal 3 cm to the following distal 3 cm segments of the human hair shaft. Distal hair segments may be exposed to external influences longer than proximal segments and thus may have a more damaged hair structure (DAWBER 1996). Therefore, the impact of external influences could be greater and the loss of cortisol could be higher with increasing distance from the skin surface. In addition to washing and shampooing hair in humans, exposure to rain or bathing in natural waters may have a similar washout effect on cortisol levels in animal hair. Furthermore, previous studies have shown a significant decrease in HCCs after exposure to natural sunlight and artificial light (GRASS et al. 2016, WESTER et al. 2016), presumably due to the enhanced degradation of cortisol, as radiant energy seems capable of directly destroying incorporated substances (GRASS et al. 2016).

2.3.5 Specific characteristics and applications of hair cortisol

Due to continuous hair growth and the associated incorporation of cortisol into the hair shaft, the cortisol level in a hair sample reflects the HPA axis activity from the entire period of hair growth (MEYER and NOVAK 2012, STALDER and KIRSCHBAUM 2012). Since hair growth lasts several months to years (MOWAFY and CASSENS 1976a, BINZ and BAUMGARTNER 2016), hair as a biological matrix may be a promising indicator for the assessment of long-term stress. Since cortisol, which is incorporated into the hair shaft, has a high stability when protected from light, hair samples can easily be stored at room temperature in the dark (RUSSELL et al., 2012). In addition, sampling hair is minimally invasive, and the procedure itself has no influence on the cortisol in the collected sample (RUSSELL et al. 2012, STALDER and KIRSCHBAUM 2012). Another special characteristic of

hair as a sampling material is the possibility of using it as a 'retrospective calendar' (KIRSCHBAUM et al. 2009). Barring cortisol-degrading influences, the continuous incorporation and stable retention of cortisol in the hair shaft during hair growth capture the average cortisol concentrations of the previous months and possibly years, depending on the hair length (KIRSCHBAUM et al. 2009). Assuming a hair growth rate of one centimetre per month in human scalp hair (BINZ and BAUMGARTNER 2016), the proximal centimetre above the skin represents the last month of hair growth, the next distal centimetre the second last month and so on (RUSSELL et al. 2012). In humans, analysing hair segments is thus increasingly common to monitor the therapy of patients with hyper- or hypocortisolism, for example (THOMSON et al. 2010, GOW et al. 2011, HODES et al., 2018) or to assess cortisol fluctuation during pregnancy (D'ANNA- HERNANDEZ et al. 2011, BRAIG et al. 2015). Research on hair cortisol in humans has focused on changes in cortisol related to mental health, including psychiatric disorders (VIVES et al. 2015, STALDER et al. 2017) and various social stressors, such as unemployment (DETTENBORN et al. 2010) and stress at school (GROENEVELD et al. 2013, MINKLEY et al. 2015). Furthermore, hair cortisol analysis is also used in the diagnosis, prognosis and therapy of other clinical conditions, such as Cushing syndrome, adrenal insufficiency and cardiovascular disease (WESTER and VAN ROSSMUN 2015, GREFF et al. 2019, IOB and STEPTOE 2019). Besides, there is first evidence that stress management can help to reduce stress and thus lower hair cortisol concentrations (IGLESIAS et al. 2015).

When investigating the impacts of different stressors on HCC, it was found that non-stress-related factors also influence the incorporation and elimination of cortisol into/from the hair shaft (STALDER et al. 2017). These influencing factors can be categorised into individual- or animal-based factors, as well as seasonal and hair-specific factors. Individual-based factors include, for example, the age and sex of the individual and the reproductive cycle in females. These factors are directly dependent on physiological fluctuations in HPA axis activity, such as increased systemic cortisol levels prior to delivery (OBEL et al. 2005). Hair-specific influencing factors can be derived from the special characteristics of hair as a biological sampling material. Due to the pigmentation of the hair and the potential light-protecting and binding properties of melanin, the colour of the hair sample can influence hair cortisol levels (STAUFENBIEL et al. 2015, BINZ et al. 2018). In addition, varied blood supply, skin temperature and external influences on different body regions can influence the HCC in the sampling region (SHARPLEY et al. 2010, LI et al. 2012).

3 Research focuses and aims

Under commercial husbandry conditions, farm animals can be exposed to various stresses and strains caused by poor housing conditions, for instance. Since stress can compromise animal welfare and lead to impaired product quality, interest is growing in the study of stress indicators and their application in monitoring programmes. Previous studies in various species have shown that hair cortisol concentration seem to be a promising retrospective marker of systemic cortisol levels and therefore a possible long-term indicator of stress. Thus, the general aims of the present work are to investigate influencing factors on HCC, and to examine the potential of hair cortisol concentration as an indicator of long-term stress in cattle and pigs. For this purpose, four studies have been conducted which build on each other (Fig. 4). First of all, the aims of **Study 1** were to present the current state of knowledge regarding the possible use of HCCs for the assessment of stress in non-human animals and to identify knowledge gaps in hair cortisol research as a basis for subsequent studies. Considering the findings of this review, **Study 2** aimed to identify the impact of the potential influencing factors on HCCs in cattle and pigs. After that, the aim of **Study 3** was to investigate whether and when long-term increased systemic cortisol levels were reflected in elevated HCCs in these species. Based on the results of Studies 2 and 3 and on hints in the literature, I assumed that in addition to the influencing factors already examined, contamination and elimination by washout may affect HCCs. Thus, **Study 4** was conducted to investigate the impact of contaminating hair with cortisol-containing body fluids and the elimination effect of water treatment on hair cortisol levels *in vitro*.

For **Study 1**, I performed extensive literature research and presented the results as a review article. Hair-specific characteristics, including the benefits and limitations of hair, were summarised and compared with other sampling materials for cortisol analysis. After an overview of the impact of stressors, various non-stress-related factors were identified that may influence the cortisol concentrations in animal hair, and practical recommendations for the use of hair cortisol were given.

Considering the findings of that review, **Study 2** focused on investigating influencing factors, including animal-based, seasonal and hair-specific variables, such as age and sex, hair colour, sampling region and hair segments. It is assumed that hair cortisol levels reflect variations in HPA axis activity and hair-specific differences due to growth, location and maturation of the hair.

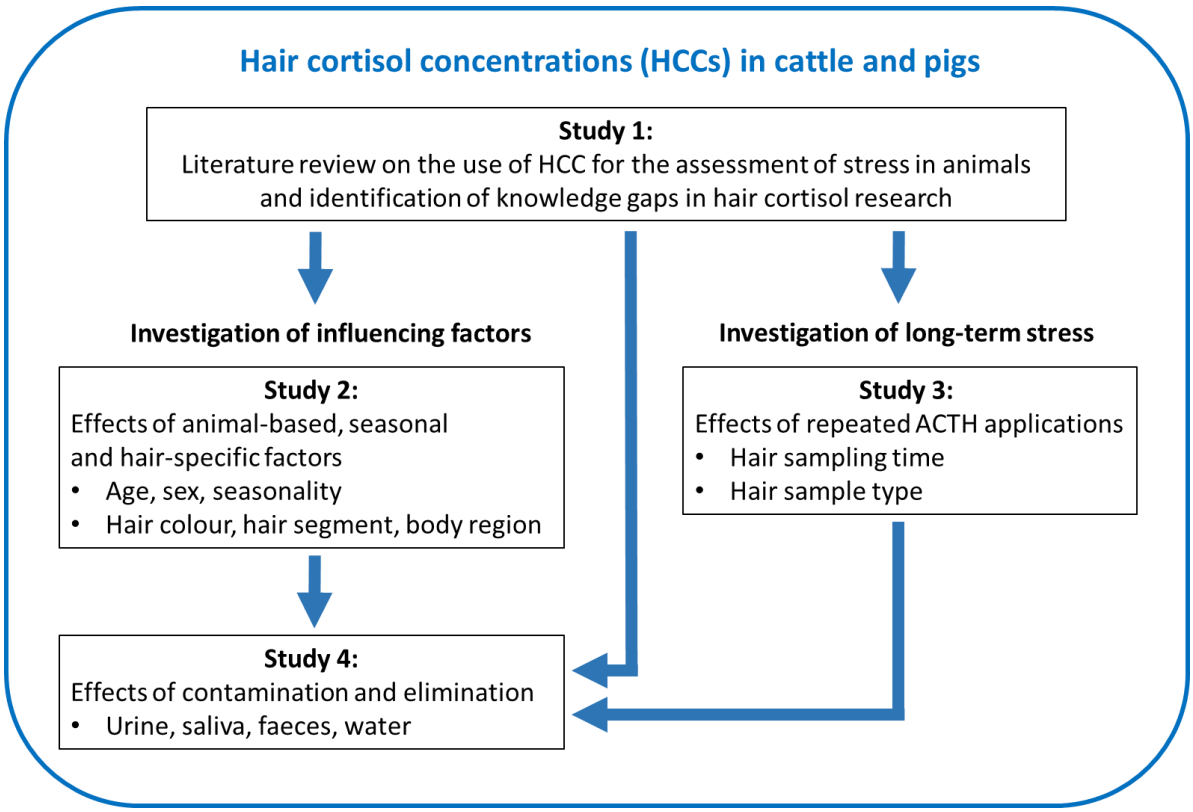

Fig. 4: Overview of the studies conducted within the present thesis

In **Study 3,** we examined the effect of long-term stress, the best time for hair sampling after a period of long-term stress and the most suitable type of hair samples. For these purposes, repeated intramuscular (i.m.) injections of ACTH were administered to induce HPA axis activity and simulate long-term stress over four weeks. The HCC analysis was performed in three different hair sample types (natural, regrown and segmented hairs) at four sampling times. Based on average hair growth rates in cattle and pigs, models for the time course of cortisol incorporation into the hair shaft were established, and we tested whether and after what time period long-term elevated systemic cortisol levels were reflected by increased cortisol concentrations in different hair sample types. It was hypothesised that repeated ACTH administrations increased cortisol concentrations in all hair sample types, with a higher magnitude in regrown hair and the highest levels within four weeks after the stress period.

As a follow-up to the findings of Studies 2 and 3**, Study 4** was conducted. In this *in vitro* study, bovine and porcine hair samples were repeatedly contaminated with urine, saliva and faeces of the respective species, treated with water or left untreated. A potential relationship between the cortisol concentration in the contaminating fluid and the amount of cortisol incorporated into the hair was considered using urine and saliva with physiologically high and low cortisol concentrations. This study was based on the hypothesis that externally contaminating hairs with body fluids increases their cortisol concentrations depending on the cortisol concentration in the fluid.

4 Results

4.1 Study 1: Hair cortisol for the assessment of stress (review)

The use of hair cortisol for the assessment of stress in animals

Susen Heimbürge, Ellen Kanitz, Winfried Otten

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Highlights:

- Hair cortisol concentration (HCC) is a useful biomarker of long-term stress.
- Minimally invasive hair sampling offers many benefits in animal-welfare research.
- Age, pregnancy, hair colour, body region, sex and season of year may affect HCC.
- Sampling protocols with a standardisation of interfering factors should be used.

Statement of contribution:

My own contribution to the first publication of my thesis comprised the conceptualisation of the manuscript, the literature research and the interpretation and summary of the findings considering their relevance. I wrote and submitted the manuscript with the support of and in agreement with the co-authors.

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The use of hair cortisol for the assessment of stress in animals

Susen Heimbürge, Ellen Kanitz, Winfried Otten*

Leibniz Institute for Farm Animal Biology (FBN), Institute of Behavioural Physiology, Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany

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ABSTRACT

The hair cortisol concentration (HCC) is assumed to be a retrospective marker of integrated cortisol secretion and stress over longer periods of time. Its quantification is increasingly used in psychoneuroendocrinological studies in humans, but also in animal stress and welfare research. The measurement of HCCs for the assessment of stress offers many considerable benefits for use in domesticated and wild animals, especially due to the easy and minimally invasive sampling procedure and the representation of longer time periods in one sample. This review aims to outline the different fields of application and to assess the applicability and validity of HCC as an indicator for chronic stress or long-term activity of the hypothalamic-pituitary-adrenal axis in wild and domesticated animals. Specific hair characteristics are presented and the advantages and limitations of using HCC are discussed. An overview of findings on the impact of stress- and health-related factors on HCCs and of diverse influencing factors causing variation in hair cortisol levels in different species is given. Recommendations for the use of hair cortisol analysis are proposed and potential fields of future research are pointed out. The studies indicate an effect of age and pregnancy on HCCs, and cortisol incorporation into hair was also found to depend on hair colour, body region, sex and season of year, but these results are less consistent. Furthermore, the results in animals show that a wide array of stressors and pathological conditions alters the cortisol concentrations in hair and that HCC thereby provides a reliable and valid reflection of long-term cortisol secretion in many species. However, more research is necessary to investigate the underlying mechanisms of cortisol incorporation into the hair and to explore the hair growth characteristics in the species of interest. To overcome confounding influences, the use of standardized sampling protocols is strongly advised.

1. Introduction

Investigations of physiological stress responses to acute or long-term stress routinely use cortisol measurement as an indicator for the activity of the hypothalamic-pituitary-adrenal (HPA) axis. Common matrices for the analysis of cortisol or its metabolites are blood, saliva, urine and faeces. In these biological materials, the measured cortisol levels represent only a retrospective timespan of a few minutes up to one or two days (Meyer and Novak, 2012; Novak et al., 2013). Thus, to assess chronic stress or long-term activity of the HPA axis, repeated and, in some cases, elaborate samplings have to be applied. Using a novel approach, Cirimele et al. (2000) analysed ten different corticoids in human scalp hair, and Koren et al. (2002) investigated cortisol concentrations in hair of rock hyrax, indicating the suitability of hair cortisol as an innovative indicator for the assessment of chronic stress. The potential benefits of hair cortisol analyses caused a rapid expansion of this research area, especially in psychoneuroendocrinological studies in humans, but also increasingly in animal stress and welfare research. In this context, the evaluation of chronic or repeated stress is particularly important, because it may cause higher biological costs of coping with the stressor, diverting resources away from other biological functions such as immune competence, reproduction or growth (Moberg, 2000; Möstl and Palme 2002). There are several reviews providing deeper insights into research using human hair samples, cortisol incorporation into the hair, sampling techniques and analytical methods (Burnard et al., 2017; Gao et al., 2016, 2013; Russell et al., 2012; Stalder and Kirschbaum, 2012). This review aims to assess the applicability and validity of hair cortisol concentrations (HCCs) as an indicator for chronic stress or long-term activity of the HPA axis in wild and domesticated animals and is the first review which outlines the various fields of application of hair cortisol analysis focused on animal species. Hair-specific characteristics are presented and the advantages and limitations of using HCC in comparison to other matrices are discussed. An overview of findings on the impact of stress- and health-related factors on HCCs in different animal species and of diverse influencing factors, such as age, sex and hair colour, causing variation in HCC is given. Finally, missing knowledge is highlighted and recommendations for the use of hair cortisol analysis and further research are proposed.

* Corresponding author.
E-mail address: otten@fbn-dummerstorf.de (W. Otten).

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2. Stress response of the HPA axis

Physiological stress responses are crucial for an organism to adapt to acute and chronic stressors. One major neuroendocrine system involved in the physiological response to stress is the HPA axis (Dallman et al., 1987). Activation of the HPA axis is accompanied by the release of glucocorticoids (GCs), which induce homeostatic processes in energy metabolism and neurobiological function (Miller and O'Callaghan, 2002; Sapolsky et al., 2000). The HPA axis comprises three major components, the hypothalamus, the anterior pituitary and the adrenal cortex. Neurons of the paraventricular nuclei of the hypothalamus synthesise corticotropin-releasing hormone (CRH), which triggers the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary into the blood (Johnson et al., 1992; Spencer and Deak, 2017). In the adrenal cortex, ACTH stimulates the production and release of GCs, with cortisol being the principal GC in most mammals and fish, and corticosterone being the major GC in birds and rodents (Mormède et al., 2007; Spencer and Deak, 2017).

There are numerous regulatory functions of cortisol throughout the body, e.g., lipolytic and proteolytic activity to mobilise energy stores, gluconeogenesis, suppression of immune reactions and neurobiological effects (Sapolsky et al., 2000). Activation of the HPA axis and the release of GCs are usually pulsatile and follow diurnal and ultradian rhythms (Mormède et al., 2007; Ralph and Tilbrook, 2016; Spiga et al., 2014). Exposure to stressors is commonly associated with increased HPA axis activity, and therefore, the response of cortisol is generally considered an indicator for stress (Dallman et al., 1987; Sapolsky et al., 2000). The magnitude of the cortisol response to acute stressors can indicate stressor intensity, and changes in the basal levels or long-term profiles were shown to be associated with chronic stress, mental health disorders and pathological conditions in humans (McEwen, 2008; Walker et al., 2013). However, there are also pitfalls that must be considered when using cortisol concentrations as a stress marker because increased HPA activity can also be caused by metabolic processes, positive affective states, mating behaviour and physical activity (Mormède et al., 2007; Ralph and Tilbrook, 2016). Commonly, cortisol and its metabolites can be measured in different sample matrices, like blood, saliva, urine and faeces (Cook, 2012). More recently, the specific characteristics of hair cortisol were examined as a potential tool for measuring the long-term activity of the HPA axis (Burnard et al., 2017; Russell et al., 2012; Stalder and Kirschbaum, 2012).

3. Hair-specific characteristics and cortisol incorporation

Information about how substances can be incorporated into the hair shaft can be derived from the multi-compartment model by Henderson (1993), but the mechanisms of cortisol incorporation are not yet fully understood (Meyer and Novak, 2012). Hair growth occurs in cycles consisting of three phases: active growth (anagen), transition (catagen) and resting (telogen) (Harkey, 1993). Systemic cortisol can be only incorporated into the growing hair shaft from blood vessels via passive diffusion during the anagen phase (Meyer and Novak, 2012). In this case, incorporation occurs in the hair follicle which is located several millimetres below the skin surface (Harkey, 1993; Udo, 1978). Thus, there is a time delay between cortisol incorporation into the hair and the time point at which this hair section arrives at the skin surface (Montillo et al., 2014; Stalder and Kirschbaum 2012). This time delay depends on hair growth velocity, may vary between species and body regions (Burnett et al., 2014; Trevisan et al., 2017) and must be taken into account when using HCC as a stress indicator.

There are several reports raising concerns about the validity of HCC to represent HPA axis activity and systemic cortisol alone. Salaberger et al. (2016) could show that hair of sheep that was brushed or treated with dexamethasone contained higher HCC compared with untreated hair. These findings indicate that GCs in the hair shaft may derive from other sources, e.g. contamination or local production by hair follicle cells (Keckeis et al., 2012; Slominski et al., 2007). Incorporation of cortisol into the hair from the blood alone was also questioned by studies in brown bears (Cattet et al., 2014; Macbeth et al., 2010). The authors found differing HCCs among different capture methods, which could not be explained by cortisol incorporation with hair growth and thus may indicate contamination by excreta, saliva, sweat, or by local cortisol sources such as skin cells or sebaceous glands.

For the use of HCC as a biomarker of stress, it is important that the sample contains enough actively growing hairs, which can be achieved by the "shave-reshave" method. A certain area is shaved at the beginning of the period of interest, and the regrown hair in the same area is reshaved at the end of this period (Davenport et al., 2008; Meyer and Novak, 2012). In addition to the hair growth cycle, which relates to single hairs, the seasonal shedding rhythm of the whole coat, different hair growth rates (depending on species, age, sex and sampling region), and possible confounding effects such as variation in skin temperature and hair colour have to be considered (Mowafy and Cassens, 1975; Sharpley et al., 2012).

Kirschbaum and colleagues showed in humans that there is a decline in HCC from the first proximal hair segments to the distal ones, the so-called washout effect (Kirschbaum et al., 2009). Explanations for these results could be UV radiation (Wester et al., 2016), hair washing frequency and hair treatment in humans (Stalder et al., 2017) or the influence of grooming in animals (Acker et al., 2018). However, other studies revealed diverging results regarding the washout effect. Carlitz et al. (2015) and Duran et al. (2017) found a decrease in HCC along the hair shaft in chimpanzees and horses, whereas in dogs, grizzly bears and orang-utans, HCC seem to be stable along the whole hair (Bennett and Hayssen, 2010; Carlitz et al., 2014; Macbeth et al., 2010).

In summary, the various hair-specific characteristics like cyclic hair growth, the time delay for sampling and a potential decline of HCC along the hair shaft have to be considered when using HCC as a stress indicator. However, there is a particular need to further elucidate the mechanisms of cortisol incorporation into the hair shaft and potential sources of contamination.

4. Comparison of matrices for analyses of cortisol and metabolites

4.1. Sampling procedure

For the evaluation of stress, it is essential that the process of sampling does not interfere with the stress marker. This could be the case with blood samples, for which animals have to be captured and restrained before sampling (Sheriff et al., 2011). In addition, a venous puncture itself is a stressful, invasive sampling method, and thus, there is growing interest in non-invasive or minimally invasive sampling techniques. Saliva sampling is minimally invasive and therefore highly practicable in various animal species. However, preceding feed or water intake may interfere saliva sampling by increased salivation or contamination with food. Collecting faeces from the environment is a non-invasive sampling method, but results usually cannot be assigned to individual animals, whereas the collection of urine is difficult and not practicable for many approaches. In comparison, the process of hair sampling is minimally invasive and painless. Moreover, it is possible to collect shed hair in sleeping nests (Carlitz et al., 2016) or abraded hair from fences without disturbance of the animals (Cattet et al., 2014). Shaving and sampling of hair is an easy procedure that can be performed after a short instruction and that, in contrast to blood sampling, does not require the presence of a professional. Furthermore, centrifugation, refrigeration and freezing of samples are not required initially (Russell et al., 2012). As a precaution, hair samples could be stored in a dry and dark place to avoid a possible washout effect by UV radiation (Wester et al., 2016). Nevertheless, hair cortisol seems to have a high long-term stability over month and years, as shown in cattle (González-de-la-Vara et al., 2011), bears (Macbeth et al., 2010) and human mummies (Webb et al., 2010).

4.2. Represented time periods of stress

The different sample matrices in which cortisol or its metabolites can be measured provide information about diverging time periods (Sheriff et al., 2011). Cortisol levels in blood and saliva represent only a short time period, and thus, repeated sampling is necessary for an integrated cortisol response and daily fluctuations have to be considered (Russell et al., 2012). In comparison, cortisol and its metabolites in urine and faeces are detectable from previous hours up to days, however with considerable species-specific differences in the time course of excretion (Möstl and Palme, 2002). During hair growth, cortisol is continuously incorporated into the hair shaft. Therefore, it is possible to assess HPA axis activity during the last weeks and months in one hair strand, depending on hair length and growth rate (Meyer and Novak, 2012). In this way, the necessity and effort of taking repeated samples can be avoided. Thus, HCC is a useful marker for long-term stress but not for short, single or scarce events (Ashley et al., 2011; Tallo-Parra et al., 2017). However, the time delay between incorporation of cortisol into the hair shaft and the appearance of this section of hair at the skin surface has to be considered. Long hair can be cut into segments, where each segment represents a proximate measure of a particular time period, and thus, a "retrospective calendar" of stressful events can be created (Russell et al., 2012). In this context, the cortisol concentrations in lanugo and vellus hair of neonates could be used for the assessment of prenatal stress (Kapoor et al., 2016).

5. Hair cortisol as a measure of stress

5.1. Experimental stimulation

Administrations of ACTH are experimentally used in various species to induce the release of cortisol from the adrenal cortex and to assess the HPA axis response (Mormède et al., 2007; Otten et al., 2004; Rushen et al., 2008). As repeated ACTH applications can mimic the HPA response to recurrent stressors (Otten et al., 2004), these experimentally induced cortisol releases were also used to validate HCC as an indicator for long-term stress. Dairy cattle, treated three times with ACTH during two weeks, showed significantly higher HCCs than saline-treated or control animals (González-de-la-Vara et al., 2011). Comparable results were found after repeated ACTH treatments over periods ranging from two weeks to more than two months in goats (Endo et al., 2018), Canada lynx (Terwissen et al., 2013) and chipmunks (Mastromonaco et al., 2014). However, HPA activation by single doses of ACTH might be insufficient to affect HCC. Ashley et al. (2011) applied a single dose of ACTH in caribou and reindeer, and Tallo-Parra et al. (2017) treated calves two times with ACTH. Both of them failed to see an effect on HCC, indicating the robustness of hair cortisol levels against occasional stress reactions. This may be due to the fact that a short section of increased cortisol in the hair shaft after a single short-term stress is not sufficient to modify HCC measured in a later sample of whole hairs. Using a slightly different approach, Schubach et al. (2016) administered CRH to Angus heifers twice daily over a period of two weeks and found significantly higher HCCs in CRH-treated animals compared with hair samples from control heifers. Together, the findings suggest that repeated stimulation of the HPA axis by administrations of ACTH or CRH is reflected by an increased accumulation of cortisol in the hair shaft. This also implies that HCC can be used for the integrative assessment of long-term HPA axis activity.

5.2. Living, housing and management conditions

It was shown in companion animals that poor living conditions affect HPA axis activity and thus HCCs. Solitary housing of dogs decreased HCC compared with dogs in multi-dog households (Bennett and Hayssen, 2010), but increased HCC compared with paired housing (Grigg et al., 2017). Nicholson and Meredith (2015) showed a positive correlation between HCC and the length of time dogs were regularly left alone, indicating a higher stress level caused by solitary housing. Additionally, it was shown that competition dogs had higher HCC than companion and professional working dogs (Roth et al., 2016). Furthermore, a number of studies in farm, zoo and wild animals have investigated the relationship between HCC and housing conditions or stressful procedures. In pigs, housing in barren conditions caused significantly higher HCCs compared with housing in enriched pens (Casal et al., 2017b), and weekly mixing of animals resulted in elevated HCCs (Casal et al., 2017a). In beef cattle, a substantial reduction in stocking density from 25,000 to 14 square metres per heifer resulted in significantly increased cortisol concentrations in tail switch hair (Schubach et al., 2017), whereas minor differences in stocking density of cattle had no effect on HCC (Silva et al., 2016). Accordingly, Dettmer et al. (2014) observed a higher HCC in rhesus macaques from high-density environments than in animals living at a lower population density. Castration of calves is a stressful and painful procedure, which leads to increased plasma and saliva cortisol concentrations (González et al., 2010; Petherick et al., 2014). In line with these findings, Creutzinger et al. (2017) showed a significantly higher HCC in surgically castrated compared with sham-castrated calves, indicating that surgical castration likewise leads to a higher HCC. However, the effect of this single stressful procedure on HCC was not consistent, as other studies reveal no significant effect in cattle (Marti et al., 2017, 2015). Capture and handling of brown bears (Cattet et al., 2014), the intervention of humans in the habitat of wild chimpanzees (Carlitz et al., 2016), heavily hunting of wolves (Bryan et al., 2015) and the relocation of monkeys (Davenport et al., 2006; Fairbanks et al., 2011; Yamanashi et al., 2016a), rabbits (Peric et al., 2017) and cows (Comin et al., 2011) from their habitual environment are also stressors that can cause an increase in HCC. Interestingly, in the study of Peric et al. (2017), not only relocation induced an increase of HCC, but also the change of employees in the facility. Farming of Asiatic black bears on bile farms causes a plethora of physical and mental sufferings (Maas, 2000) and thus, unsurprisingly, relocation of these bears from a bile farm to a rehabilitation facility was accompanied by a decline in HCC (Malcolm et al., 2013). In summary, a wide range of different stressors associated with the housing, management and handling of animals increase HPA axis activity, which can be reflected by an increased HCC, especially when the period of stress experience covers weeks or months.

5.3. Social behaviour

The social environment of animals and stress induced by social interactions and dominance are important modulators of HPA axis activity (Creel et al., 2013). Whether social stress affects dominants or subordinates to a greater degree depends on the species- and sex-specific allostatic load of social status, which may be assessed by GC profiles in blood, urine and faeces (Goymann and Wingfield, 2004). Likewise, the relationship between social rank and hair cortisol concentrations is ambiguous between species. Koren et al. (2008) reported that singing rock hyrax, which are more dominant, show a higher HCC in comparison with non-singing males. On the other hand, in female rhesus macaques living in less stringent groups, elevated HCCs were found in low-ranking compared with higher ranking monkeys (Qin et al., 2013). It was shown in lemurs and chimpanzees that receiving aggressions like chasing, hitting or biting, also relates to a higher HCC (Tennenhouse et al., 2017; Yamanashi et al., 2018, 2016b, 2013). Furthermore, Finkler and Terkel (2010) found a significant positive correlation between agonistic behaviour and hair cortisol levels in female cats, indicating that aggressive cats had higher HCCs. Animal temperament may be an additional personality trait affecting HCC, as shown in chipmunks where HCC was positively correlated with docility (Martin and Réale, 2008), but no relationship between temperament and HCC was found in cattle (Cooke et al., 2017; Lockwood et al., 2017). In conclusion, social environment and behaviour can affect HCC,

but the outcome is closely linked to species-specific and individual characteristics.

5.4. Body condition and nutritional status

Several studies also investigated hair cortisol as a measure of nutritional stress and body condition in different species. In this regard, Bryan et al. (2013) analysed cortisol in hair from salmon-eating grizzly bears and found a significant negative correlation between HCC and salmon availability. Likewise, Macbeth et al. (2012) and Cattet et al. (2014) showed that HCCs in polar bears and free-ranging brown bears are negatively associated with body condition. Also in pigs, it was shown that lean sows had higher HCCs compared with normal-weight sows (Trevisan et al., 2017). The effects of water restriction during heat-stress conditions on cortisol levels in the blood and hair of sheep were investigated by Ghassemi Nejad et al. (2014). HCC was higher in animals that suffered a 3-hour water restriction after feeding than in sheep that had free access to water or only a 2-hour water restriction. These findings suggest that HCC may be a useful indicator of water restriction or nutritional stress caused by increased foraging behaviour and/or mobilisation of energy stores during periods of reduced food availability

5.5. Diseases and disorders

The appearance of medical disorders and clinical diseases can be accompanied by altered activity of the HPA axis. As shown in sheep, infection of the right hind foot with ovine footrot caused a decrease in HCC in both limbs (Stubsjoen et al., 2015). A study in eastern grey kangaroos described a significantly higher HCC in animals infected with lumpy jaw disease compared with healthy control animals, however, this effect was dependent on body region where hairs were sampled (Sotohira et al., 2017). As might be expected, HCC in dogs with Cushing syndrome was higher than in healthy controls, indicating that HCC can be a helpful tool for the diagnosis and therapy of hypercortisolism and adrenal insufficiency (Corradini et al., 2013; Ouschan et al., 2013). Studies in rhesus macaques showed a positive correlation of HCC with hair loss, although there were no indices of concomitant stressors causing the alopecia (Lutz et al., 2016; Novak et al., 2014). Likewise, a significantly higher HCC was observed in dogs with atopic dermatitis compared with healthy dogs (Park et al., 2016). The positive correlation of HCC with the extent of cutaneous lesions indicates that hair cortisol may be increased as a consequence of chronic physical discomfort caused by itchy, dry and inflamed skin in dogs (Park et al., 2016). Infections with gastro-intestinal parasites, however, revealed no significant effects on HCC in reindeer (Carlsson et al., 2016) or pigs (Trevisan et al., 2017).

In addition to the previous studies, which were focused on a certain disease, health status per se as measured by the occurrence of different clinical diseases was found to elevate HCC in cows (Burnett et al., 2015; Comin et al., 2013). Expectedly, this increase depends on the duration of the disease because a higher HCC was found in chronically ill compared with acutely ill animals (Braun et al., 2017a). Additionally, mental disorders can also affect HCC in non-human primates. Qin et al. (2015) showed that rhesus macaques that spent longer times in huddle posture, which is interpreted as a sign of depression, had a significantly higher HCC than those who huddled for shorter periods of time. In summary, the majority of studies revealed that the appearance of medical disorders and diseases can be accompanied by an increase in HCC; however, elevated HCC may only indicate the occurrence of but not the type of disorder.

6. Factors influencing hair cortisol concentrations

For the use of HCC as a marker of long-term stress, it is important to consider its determinants, which may confound results and need to be controlled for when making comparisons between, but also within individuals or groups. Potential influencing factors comprise, for example, age, sex, pregnancy, season of year, and hair-specific features such as hair colour or body region from which the sample is taken.

6.1. Age

Elevated cortisol levels were found in hair samples obtained from 15-day-old calves compared with those from 2-year-old cows (González-de-la-Vara et al., 2011) and in new-born foals compared with foals at 30 or 60 days of age (Comin et al., 2012a; Montillo et al., 2014). Additionally, infant and juvenile non-human primates show higher circulating levels of GCs than adults, and this is similarly reflected in an age-related decline in HCC (rhesus monkeys: Dettmer et al., 2014; vervet monkeys and baboons: Laudenslager et al., 2012; Fourie and Bernstein, 2011; further primates: Fourie et al., 2016). This may be caused by lower corticosteroid binding globulin concentrations in infants, resulting in increased plasma concentrations of free cortisol as shown in humans (Grant et al., 2017; Gunnar and Donzella, 2002). However, it was also found in baboons that HCC can increase again later in life (Fourie et al., 2015). When different age groups were only compared within juvenile or adult animals no differences in HCC were found, as shown in orang-utans (Carlitz et al., 2014) and dogs (Roth et al., 2016). Together, these findings indicate that there is an age-dependent decline in HCC from young to adult age groups. However, the precise time course seems to be species-specific and may even comprise a later increase at older ages.

6.2. Sex

Findings in American black bears (Lafferty et al., 2015) and coyotes (Schell et al., 2017) indicate higher HCCs in males than in females. Increasing testosterone levels in adolescent males are associated with the manifestation of reproductively relevant behaviours, which themselves may be experienced as stressful events and may result in elevated cortisol concentrations (Bergman et al., 2005). Furthermore, higher cortisol levels in males may be caused by lower activity of the glucocorticoid-metabolising enzyme 11β-hydroxysteroid dehydrogenase 2, as shown in humans (Raven and Taylor, 1996). In contrast, studies in polar bears (Bechshoft et al., 2011), brown bears (Cattet et al., 2014) and non-human primates (Dettmer et al., 2014; Fourie et al., 2016; Laudenslager et al., 2012) showed significantly higher HCCs in females than in males. This may be partly explained by sex differences in the body condition index (Cattet et al., 2014) or in sex-specific effects of gonadal steroids on basal and stress-induced HPA axis activity (Laudenslager et al., 2012; Veldhuis et al., 2013). Accordingly, castrated female cats had lower HCCs than intact cats (Finkler and Terkel, 2010). In addition, studies in Asiatic black bears (Malcolm et al., 2013), Canada lynx (Terwissen et al., 2013), chimpanzees (Yamanashi et al., 2013) and orang-utans (Carlitz et al., 2014) failed to find significant differences between sexes. In summary, the influence of sex on HCC seems to be inconsistent. A diverging cortisol secretion between males and females may depend on numerous factors such as different behavioural pattern, body condition and metabolism of gonadal steroids.

6.3. Pregnancy

Cortisol plays an important role in the maturation of foetal organ systems and the induction of parturition (Challis et al., 2001), and previous studies in many species have shown an increase in circulating cortisol with progressing pregnancy until delivery (Edwards and Boonstra, 2018; Obel et al., 2005). In vervet monkeys, a significant positive correlation of HCC with the month of pregnancy could also be shown. Females sampled within one month after delivery had significantly higher HCCs than did females sampled earlier in pregnancy or non-pregnant females (Fairbanks et al., 2011). Bacci et al. (2014)

reported higher hair cortisol levels during late pregnancy and lactation in sows than during early-mid pregnancy. In cows, HCC remained largely unchanged during pregnancy but increased significantly in the month of parturition (Braun et al., 2017b). The few studies so far in different animal species show a general increase of HCC during pregnancy.

6.4. Season of year

Various studies in different species show inconsistent results for seasonal influences. Higher HCCs during winter and lower ones during summer were observed in pigs (Bacci et al., 2014) and dogs (Roth et al., 2016). Martin and Réale (2008) reported higher cortisol levels in hair from chipmunks harvested in summer compared with spring. In brown bears, the cortisol levels tended to be greater in hair samples obtained from late summer and fall than in samples from spring (Cattet et al., 2014). Hair cortisol concentrations in cows deriving from regions with different temperatures generally increased from spring to summer and were lower from late summer until fall. However, the rise in cortisol levels from spring to summer appeared to be more intense in the cold-temperate region than in the warm-temperate region (Uetake et al., 2018). Additionally, a seasonal increase in sexual and territorial behaviour may induce stress and increase HCC as shown in deer bucks (Ventrella et al., 2018). Together, these findings suggest that HCC may reflect seasonal differences in behaviour and environmental impact, such as that of temperature, daylight period and food availability.

6.5. Hair colour

Studies in cattle (Burnett et al., 2014; González-de-la-Vara et al., 2011) and chimpanzees (Yamanashi et al., 2013) revealed a higher HCC in white hair than in black hair. Similarly, in a dog study conducted by Bennett and Hayssen (2010), a lower HCC was observed in black than in yellow samples. Tallo-Parra et al. (2015) found higher hair cortisol levels in black hair compared with white hair of the same cow, however black hair samples derived from different body regions, which may confound the results. Other studies in cattle failed to find significant differences between different-coloured hairs (Ghassemi Nejad et al., 2017; Nedić et al., 2017). Reasons for the contradictory findings on HCC in dark and bright hair are not totally understood but could be related to physical space within the hair shaft, increased blood flow in skin covered by black hair, interactions with melanin or higher washout in darker hair due to UV radiation (Burnett et al., 2014; Gratacós-Cubarsí et al., 2006; Neumann et al., 2017; Pragst and Balikova, 2006). Collectively, studies on the influence of hair colour on HCC show inconsistent results and the underlying mechanisms of cortisol incorporation into different-coloured hairs require further investigations.

6.6. Body region

Numerous studies in wild and domesticated animals revealed differences in HCC depending on body region (caribou and reindeer: Ashley et al., 2011; chimpanzees: Carlitz et al., 2015; Yamanashi et al., 2013; marmots: Acker et al., 2018; kangaroos: Sotohira et al., 2017; Canada lynx: Terwissen et al., 2013; cattle: Burnett et al., 2014; Moya et al., 2013; pigs: Casal et al., 2017a,b; horses: Duran et al., 2017). Indeed, other studies in rabbits (Comin et al., 2012b), bears (Macbeth et al., 2012), reindeer (Carlsson et al., 2016) and coyotes (Schell et al., 2017) failed to find region-specific variations of HCC. One possible explanation for differences in HCC between body regions could be a varying proportion of hair follicles in the anagen, catagen and telogen phases caused by cyclic hair growth and the shedding rhythm in animals. Cortisol is mainly incorporated into the hair during the anagen growth phase. Thus, higher HCCs would be found in body regions with more follicles in the anagen phase or with follicles having a longer anagen phase (Burnard et al., 2017). Additionally, washout by different weather exposure, contamination by faeces, grooming, and differing hair growth rates and skin blood flow may be potential causes for region-specific differences (Acker et al., 2018; Burnett et al., 2014; Carlitz et al., 2015; Casal et al., 2017a; Moya et al., 2013). Although quantitative differences in HCC exist between body regions in many species, comparisons between subjects are possible as long as the sampling site is not varied (Burnard et al., 2017).

7. Conclusions and future directions

The measurement of hair cortisol for the assessment of stress offers many considerable benefits for use in animals, especially due to the easy and minimally invasive sampling procedure and the representation of longer time periods in one sample. It has been shown that a wide array of stressors and pathological conditions alter the cortisol concentrations in hair and that HCC thereby provides a reliable and valid reflection of long-term cortisol secretion in many species. Therefore, hair cortisol concentrations may be used as a helpful indicator in animal welfare research. However, there are various factors influencing HCC. There is a distinct effect of age and pregnancy on hair cortisol levels, and it was also found that cortisol incorporation may depend on hair colour, body region, sex and season. However, the latter factors are inconsistent and may be caused by species-specific differences. Thus, for investigations of stress effects, hair sampling protocols should be used which standardise these interfering factors as far as possible. Recommendations for sampling and use of hair in cortisol analysis may comprise for example (1) the use of animals with the same age group, sex and reproductive state; (2) the sampling of hairs from the same body region and colour; (3) the consideration of the time delay between cortisol incorporation and sampling of hair; (4) the avoidance of external contaminations and (5) the use of the "shave-reshave" method if possible. However, more research is necessary to elucidate the influence of possible interfering factors, to investigate the underlying mechanisms of cortisol incorporation into the hair shaft and to explore the hair growth characteristics in the species of interest. In addition, the measurement of hair cortisol concentrations opens up new avenues for stress research, e.g., the use of hair segments as a retrospective calendar and of neonatal hair for the assessment of prenatal stress.

Declarations of interest

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4.2 Study 2: Effects of animal-based, seasonal and hair-specific factors on hair cortisol concentrations

Within a hair's breadth – Factors influencing hair cortisol levels in pigs and cattle

Susen Heimbürge, Ellen Kanitz, Armin Tuchscherer, Winfried Otten

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Highlights:

- Various factors may influence hair cortisol concentration (HCC) in pigs and cattle.
- HCC is affected by body region, age, hair colour and season of sampling.
- Distal hair segments exhibit higher HCCs in both species.
- There were no differences in HCC between the sexes.

Statement of contribution:

For the second publication, I designed and performed the experiments in consultation with the co-authors of the manuscript and with the support of technicians at the Institute of Behavioural Physiology. I analysed the data and presented and interpreted the results. The final manuscript and figures were prepared with the support of and in agreement with the co-authors.

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Within a hair's breadth – Factors influencing hair cortisol levels in pigs and cattle

Susen Heimbürge[a], Ellen Kanitz[a], Armin Tuchscherer[b], Winfried Otten[a,*]

[a] Leibniz Institute for Farm Animal Biology (FBN), Institute of Behavioural Physiology, Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany
[b] Leibniz Institute for Farm Animal Biology (FBN), Institute of Genetics and Biometry, Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany

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ABSTRACT

During the last two decades, hair cortisol concentration (HCC) has proven to be a promising marker for the evaluation of increased hypothalamic-pituitary-adrenal axis activity caused by repeated or long-term stressful conditions. A minimally invasive sampling procedure, simple storage and the retrospective characteristic of one hair sample are reasons why HCC is increasingly used not only in human medicine but also in animal welfare research. However, before applying HCC as a reliable indicator for stress, it is important to investigate potential influencing factors in addition to stressors in the species of interest. Thus, the aim of our study was to elucidate the impact of age, sex, hair color, body region, age of hair segments and season of hair sampling on HCC in pigs and cattle. Hair samples were taken by electric clippers and analyzed by ELISA after extraction. Our results show similar effects of influencing factors in both species. Significantly increased HCCs were found in young animals after birth compared with older age groups. In addition, HCCs were significantly higher in samples obtained from the tail tip in comparison with samples from the shoulder, neck and back regions, in black hair compared with white hair and in distal hair segments. Season had an impact on HCC only in cattle, which exhibited higher levels in winter than in summer. In conclusion, age, body region, hair color, hair segment and season affect hair cortisol concentrations and should be considered and controlled for when HCC is applied as a potential stress indicator in pigs and cattle. In addition, further research is necessary to elucidate the mechanisms by which cortisol is incorporated into the hair shaft.

1. Introduction

The physiological response to stress is associated with enhanced activation of the hypothalamic-pituitary-adrenal (HPA) axis, which triggers an increased release of glucocorticoids from the adrenal cortex, such as cortisol (Dallman et al., 1987). Thus, elevated cortisol concentration is routinely used as an indicator of stress in many species (Sheriff et al., 2011). During the last two decades, analyses of hair cortisol concentrations (HCCs) have increasingly gained importance for the assessment of long-term stress in humans (Stalder et al., 2017; Greff et al., 2019) and animals (Burnard et al., 2017; Heimbürge et al., 2019). In particular, farm animals undergo many potentially stressful situations under commercial husbandry conditions, e.g., due to confined housing environments and frequent management procedures (Fraser et al., 2001). As a result, there is growing interest in using animal-based welfare indicators to improve the wellbeing of farm animals. In this context, HCC could be a potential marker for increased stress in farm animals. However, previous studies on HCCs in diverse animal species revealed various sources of variation in hair cortisol levels in addition to stressors. These influencing factors comprise animal-based (e.g., age), external (e.g., season) or hair-specific (e.g., age of hair segment) factors, which may confound results and need to be controlled for when making comparisons between, as well as within, individuals or groups (Heimbürge et al., 2019). Thus, it is necessary to evaluate the effects of potential influencing factors on HCCs in the species of interest before applying HCC as a possible stress indicator. Because only a small number of studies investigated interfering factors on HCC in pigs and cattle (e.g., pigs: Bacci et al., 2014; Casal et al., 2017; cattle: Burnett et al., 2014; Uetake et al., 2018), there is still a need to clarify their impact in these species. Therefore, the aim of this study was to investigate the effects of variation in individual and contextual factors, such as age and sex of the animals, hair color, body region, age of hair segments and season of hair sampling, on HCCs in pigs and cattle.

2. Materials and methods

2.1. Animals and sampling procedure

All animals in this study were housed and fed according to the

* Corresponding author.
E-mail address: otten@fbn-dummerstorf.de (W. Otten).

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relevant German directives for their particular age and performance. The procedures used in this study for animal handling and hair sampling complied with the German Animal Protection law and were approved by the relevant authority (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei, Mecklenburg-Vorpommern, Germany; LALLF M-V/AZ 7221.3-1.1-059/17).

We studied the effects of different body regions and hair segments on HCC in German Landrace pigs and the effect of hair color on HCC in Saddleback pigs. Animals from both breeds were born and raised in the Experimental Pig Unit of the Leibniz Institute for Farm Animal Biology (FBN Dummerstorf). For the evaluation of age, sex and season, we used crossbred sows (German Landrace (GL) X German Large White (GLW)) and their litters ((GLxGLW) × Pietrain) from a commercial pig farm situated nearby. All pigs were housed indoors. Additionally, studies were performed in Holstein Friesian cattle kept in the Experimental Cattle Unit of the FBN Dummerstorf or the neighboring Landesgut Dummerstorf under loose housing conditions with free stalls. When samples were taken from sows and cows, animals within the same reproductive stage were used.

Hair samples were carefully clipped by an electric hair clipper (Wahl Professional KM 10, Mosonmagyaróvár, Hungary) as close as possible to the skin (trimmer blade: Oster Cryogen-X Nr. 50 (0.2 mm), Boca Raton, USA) and collected by hand. Dirty areas were avoided if possible. In the case of very short hair (e.g., regrown hair), samples were collected using a hand-held vacuum cleaner (cleanmaxx zyklon PC-P006EA, DS Produkte GmbH, Gallin, Germany) with a paper filter in the suction tube. Hair samples were dried, weighed and stored protected from light at room temperature (RT) until analysis.

2.2. Body region

For the evaluation of HCCs in different body regions, we used a total of 46 sows (mean ± SD age: 17 ± 11 months) and 40 cows (mean ± SD age: 62 ± 21 months). From both species, hair samples were shaved from the back and the tail tip, as well as from the neck region, in pigs and from the shoulder region in cattle (Fig. 1A, B).

For the determination of the hair growth rate in these regions, previously shaved areas were shaved again after 6–10 weeks in 16 sows and 12 cows. From cows, only black hair samples were collected. The hair length of 30 single hairs per animal per body region was measured with a decimal Vernier caliper, and the mean hair growth rate was calculated as mm per month (30 days).

2.3. Age and sex

In pigs, the effects of age and sex were evaluated in 36 animals (18 castrated males and 18 females) from three age groups: 2, 10 and 27 weeks of age and 18 sows (mean ± SD age: 19 ± 3 months). In cattle, the effect of age was investigated by comparing HCC in female animals across four different age groups, including newborn calves (n = 19), 6-month-olds (n = 18), 18-month-old cattle (n = 18) and cows (n = 46, mean ± SD age: 56 ± 20 months). The effect of sex was evaluated in 40 newborn calves (21 males and 19 females). From all animals, a hair sample was taken from the back as described above.

2.4. Hair color

A total of 36 Saddleback piglets (4–5 weeks of age, 13 males and 18 females) and 36 cows (mean ± SD age: 61 ± 15 months) were used to investigate the effect of hair color on HCC. One sample of black hair and one of white hair were shaved from each animal. Due to the specific coat pattern of Saddleback pigs, the black hair sample was collected from the back, whereas the white hair sample originated from the chest. In cattle, all samples were collected from the back.

2.5. Hair segment

In pigs, hair segments were sampled from 22 sows (mean ± SD age: 14 ± 9 months). Four hair segments were taken from the back by an electric clipper with an adjustable cutting length (hair clipper series 1000 QC5005/10, Philips, Drachten, Netherlands) and using a vacuum cleaner with a paper filter for sampling. Initially, hair was shaved at a length of 3 cm, with this sample representing the distal part longer than 3 cm. Thereafter, the remaining proximal 3 cm of the hair were shaved in repeated portions by reducing the cutting length by 1 cm each (Fig. 2). HCC was analyzed separately for each segment.

In cattle, the effect of the age of hair segments on HCC was investigated by taking hair strands from the tail tips of 20 cows (mean ± SD age: 63 ± 23 months). Hair strands were shaved close to the skin, and afterwards, the proximal 6 cm of each strand was cut into three 2-cm segments. HCC was analyzed separately for each segment.

2.6. Seasonality

To investigate the effect of seasonality, we used a total of 75 sows (mean ± SD age: 17 ± 4 months) and 72 cows (mean ± SD age: 64 ± 17 months). Sows were housed indoors and kept in commercial insemination pens on the day of sampling. All cows were lactating and kept in loose housing with free stalls. Hair samples were taken from 37 sows and 37 cows in July 2017 and from an additional 37 sows and 35 cows in January 2018. Sampling days were chosen at approximately 4–5 weeks after summer and winter solstice to ensure that hair samples were grown around the time of the longest and shortest daylight hours. Hair samples were collected from the back region as described above.

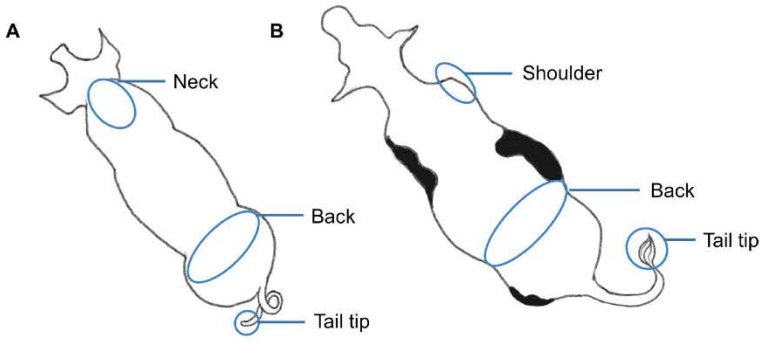

Fig. 1. Sampling regions in pigs (A) and cattle (B). Neck: dorsal line of the cervical spine; shoulder: region over the spina scapulae; back (pigs): dorsal region of the musculus gluteus maximus; back (cattle): area over the transverse process of the lumbar vertebrae; tail tip: distal end of the tail.

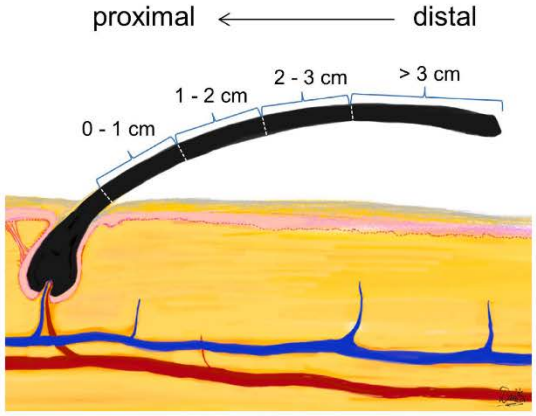

Fig. 2. Hair segments in pigs. Segmentation of porcine hair was achieved first by cutting the distal hair section longer than 3 cm (> 3 cm) and then 1 cm segments from distal to proximal (2–3 cm, 1–2 cm, 0–1 cm).

2.7. Analysis of cortisol concentration in hair

Hair samples of pigs and cattle were processed as previously described by Meyer et al. (2014) with some modifications. Each hair sample was placed in a 15-ml screw-cap polypropylene tube, then 5 ml isopropanol was added, and the tube was gently mixed on a rotator at RT for 3 min. After decanting, the wash cycle was repeated. The hair samples were then dried for approximately 2–3 days in clean protected dishes at RT. We transferred 200 mg of each sample in 10-ml stainless steel grinding jars with two 12-mm stainless steel grinding balls, snap-frozen in liquid nitrogen and pulverized using a Retsch ball mill (MM 400, Retsch GmbH, Haan, Germany) for 2 min at 30 Hz. Approximately 50 mg of pulverized hair was weighed out and transferred into a 2-ml microcentrifuge tube. One milliliter of methanol (HPLC grade) was added, and the tubes were incubated at RT for 18–24 h with slow shaking to extract the steroids. Following extraction, samples were spun in a microcentrifuge at 12,000 rpm for 2 min, and 0.6 ml of each methanol extract was aliquoted into a 1.5-ml tube and then vaporized using a vacuum evaporator (SC210A SpeedVac® Concentrator, Thermo Fisher Scientific Inc., Waltham, MA, USA). The dried extracts were reconstituted with 0.4 ml phosphate buffer provided in the assay kit. Hair cortisol concentrations were analyzed in duplicate using a commercially available ELISA kit designed for free cortisol quantification in saliva (DES6611, Demeditec Diagnostics GmbH, Kiel, Germany) according to the instructions of the manufacturer. The resulting values were converted from ng/ml to pg/mg for data analysis. The detection limit of our procedure is 1.1 pg/mg based on the lowest standard provided in the assay kit. The intra- and interassay coefficients of variation were 4.3% and 7.8% for cortisol analyses in hair of cattle and 4.5% and 6.8% for hair cortisol in pigs, respectively.

2.8. Statistical analyses

Statistical analyses were performed using the SAS System for Windows, version 9.4 (Copyright, SAS Institute Inc., Cary, NC, USA). All data were analyzed using the MIXED model procedure for performing analyses of variance (ANOVA) with hair cortisol concentration or, in one case, hair growth rate as the dependent variable. The experiments differed in fixed factors and covariates, as shown in detail in Table 1. When two fixed factors were used, their interaction was also included in the model. Repeated measures on the same animal were taken into account by the repeated statement of the MIXED procedure using a compound symmetry structure of the block diagonal residual covariance matrix. Multiple pairwise comparisons were made using the Tukey-Kramer test. Statistical tests were considered to be significant if $p < 0.05$. Results are presented as least squares means (LSM) ± standard error (SE).

3. Results

3.1. Body region

There was a significant effect of body region on the hair growth rate in both species (both: $p < 0.001$). Pairwise comparison indicated a significantly higher hair growth rate at the tail tip in comparison to hair in the neck and back regions of pigs (both: $p < 0.001$, Fig. 3A) and compared to hair in the shoulder and back regions of cattle (both: $p < 0.001$, Fig. 3B). In addition, in cattle, hair located in the back grew faster than hair in the shoulder region ($p < 0.001$, Fig. 3B).

Further, ANOVA revealed a significant effect of body region on HCC in pigs and cattle (both: $p < 0.001$). Pairwise comparisons indicated that cortisol concentrations in hair from the tail tip were significantly higher compared to cortisol concentrations in hair from the back and neck in pigs (both: $p < 0.001$, Fig. 3C) and compared to cortisol levels in hair from the back and shoulder region in cattle (both: $p < 0.001$, Fig. 3D). In addition, in pigs, HCCs were higher in the back compared to the neck region ($p < 0.01$, Fig. 3C).

3.2. Age and sex

The results of the ANOVA revealed a significant influence of age group on hair cortisol levels in both species (both: $p < 0.001$). In pigs, pairwise comparisons indicated highest hair cortisol concentrations in piglets in comparison to 10-week-old pigs, 27-week-old pigs and sows ($p < 0.001$).

Table 1
Fixed factors and covariates used in the statistical models for "hair growth rate" and "hair cortisol concentration" (influencing factors of main interest: underlined; covariates: *italic*). When two fixed factors were used, their interaction was also considered.

Dependent variable	Pigs		Cattle	
	Fixed factors	Covariates	Fixed factors	Covariates
Hair growth rate	Body region	·	Body region	·
Hair cortisol concentration	Body region, Replicate	*Litter number*	Body region, Hair color	*Sampling day, Lactation number*
Hair cortisol concentration	Age group	·	Age group, Hair color	·
Hair cortisol concentration	Sex, Age group	·	Sex, Hair color	·
Hair cortisol concentration	Hair color, Sex	·	Hair color	·
Hair cortisol concentration	Hair segment	·	Hair segment	·
Hair cortisol concentration	Season	·	Season,, Hair color	·

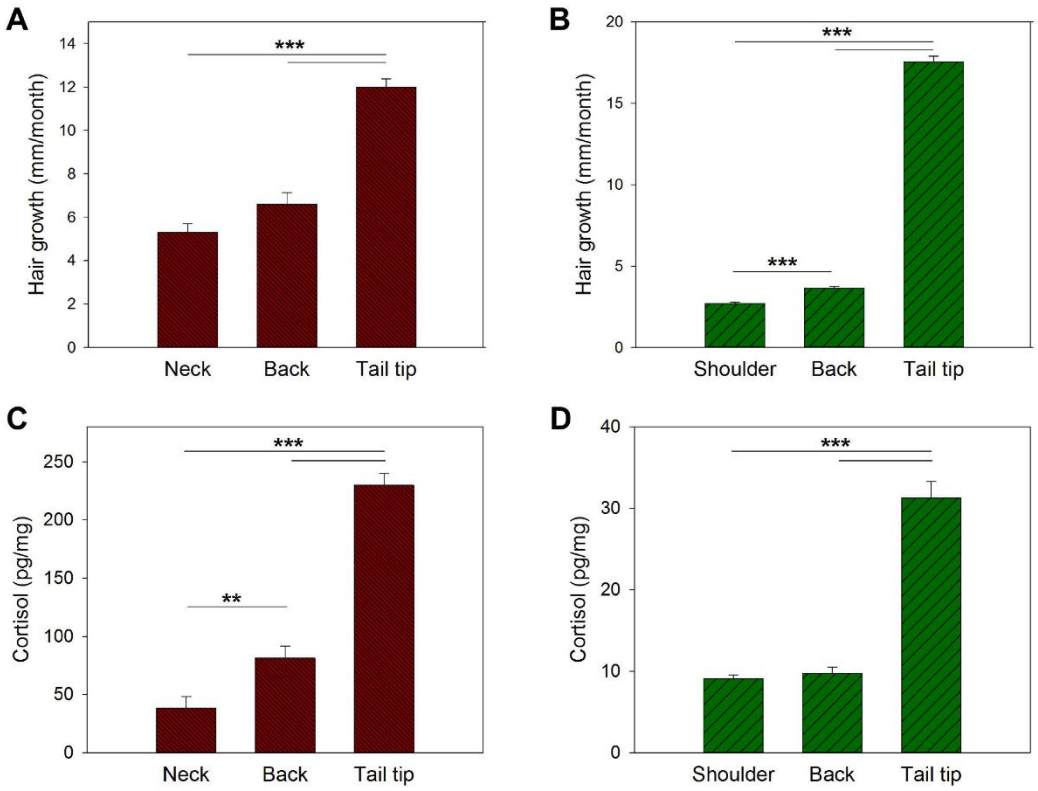

Fig. 3. (A and B) Effect of body region on hair growth rate in pigs (A) and cattle (B). (C and D) Effect of body region on hair cortisol concentrations in pigs (C) and cattle (D). Data are presented as LSM ± SE, and significant differences are indicated by asterisks (***p < 0.001, **p < 0.01).

Additionally, adult sows showed significantly higher HCC than 10-week-old pigs ($p < 0.01$, Fig. 4A). In cattle, the highest HCCs were found in newborn calves compared to cortisol levels in 6-month-old, 18-month-old and adult cattle ($p < 0.001$, Fig. 4B). The hair color of different cattle and the interaction of age group and hair color did not influence HCC.

There was no significant effect of sex of the animals on hair cortisol concentrations, neither in pigs (males: 93.7 ± 6.0 pg/mg, females: 102.5 ± 6.0 pg/mg, $p = 0.31$) nor in cattle (males: 48.4 ± 2.9 pg/mg, females: 51.7 ± 2.9 pg/mg, $p = 0.43$). The interaction of sex and age group in pigs and sex and hair color in cattle did not influence HCC.

3.3. Hair color

In both species, the statistical analysis revealed a significant influence of hair color on HCC within animals (pigs: $p < 0.05$, cattle: $p < 0.001$). Pairwise comparison revealed higher HCCs in black hair

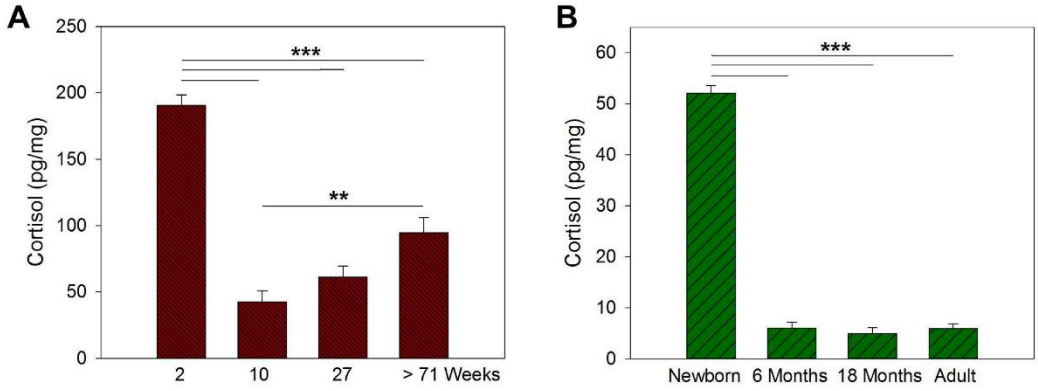

Fig. 4. Effect of age group on hair cortisol concentrations in pigs (A) and cattle (B). Data are presented as LSM ± SE, and significant differences are indicated by asterisks (***p < 0.001, **p < 0.01).

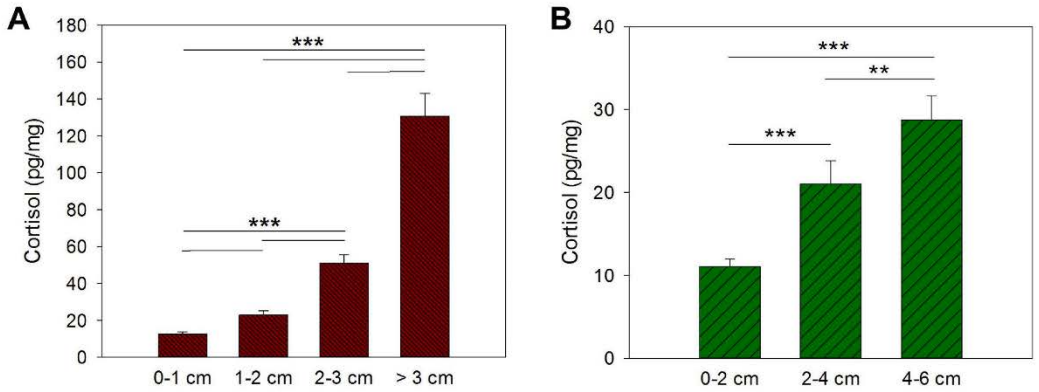

Fig. 5. Effect of hair segments on hair cortisol concentrations in pigs (A) and cattle (B). Data are presented as LSM ± SE, and significant differences are indicated by asterisks (***$p < 0.001$, **$p < 0.01$).

compared to white hair of the same animal in pigs (black: 66.9 ± 4.8 pg/mg, white: 60.2 ± 3.8 pg/mg, $p < 0.05$) and cattle (black: 8.5 ± 0.3 pg/mg, white: 7.2 ± 0.3 pg/mg, $p < 0.001$). Sex and the interaction of hair color and sex did not influence HCC in pigs.

3.4. Hair segment

ANOVA indicated a significant effect of hair segment on HCC in pigs and cattle (both: $p < 0.001$). Pairwise comparisons revealed a significant increase in hair cortisol levels from proximal to distal hair segments in both species (Fig. 5A, B).

3.5. Seasonality

There was no significant effect of season on HCC in pigs (summer: 76.4 ± 8.0 pg/mg, winter: 67.9 ± 8.0 pg/mg, $p = 0.46$). However, in cattle, there was a significant influence of season with higher HCC in winter samples compared to summer samples (winter: 8.6 ± 0.3 pg/mg, summer: 6.2 ± 0.3 pg/mg, $p < 0.001$). Additionally, in cattle, ANOVA indicated a significant effect of hair color on HCC with higher HCC in black hair samples compared to white samples ($p < 0.05$). The interaction of season and hair color was not significant.

4. Discussion

To the best of our knowledge, this is the first extensive study on factors influencing hair cortisol levels in pigs and cattle. Our results show that different ages, hair segments, body regions and hair colors significantly affect HCCs in pigs and cattle, whereas sex did not influence HCCs. The season of sampling was a source of variation in HCC for cattle only.

The cortisol levels in porcine hair in our study were generally higher than those found in previous studies in pigs (e.g., Nannoni et al., 2018; Roelofs et al., 2019), whereas HCCs in cattle hair were similar to concentrations found in other studies (e.g., Burnett et al., 2015; Ghassemi Nejad et al., 2017). It is known that diverging levels could be caused by different methodological processing and analytical methods (Greff et al., 2019) as well as by different breeds or ages of the animals or by different sampling regions (Peric et al., 2013; Dulude-de Broin et al., 2019).

4.1. Body region

In our study, hair growth rates ranged from 5.3 to 12.0 mm/month in pigs and from 3.5 to 17.0 mm/month in cattle, with highest values being observed at the tail tip. In pigs, growth rates in the neck and the back region were similar to those found previously in the rump region of sows (Bacci et al., 2014). In cattle, our results revealed slightly elevated growth rates than those found by Burnett et al. (2014), who also revealed faster growth of hair in the tail tip compared to hip and shoulder regions. Studies in primates showed average growth rates from 7 mm to 27 mm/month, depending on the species (Fourie et al., 2016; Tennenhouse et al., 2017). These differences may be caused by diverging hair types, such as hairs of different lengths, or with a different blood supply to the skin in the examined body regions. The knowledge of hair growth velocities is important for the interpretation of HCC results to enable the assessment of recent causes of variation, especially when using HCC as a retrospective calendar. Thus, it is advisable to determine the hair growth rate in different body regions of the species of interest.

Our findings regarding the impact of body region on hair cortisol levels are in accordance with results from a study in pigs, in which lower HCCs were found in cranio-dorsal regions than in dorso-lumbar areas (Casal et al., 2017). Increased cortisol levels in tail hair were also found in cattle (Burnett et al., 2014; Moya et al., 2013), whereas in horses, higher HCCs were found in mane hair than in hair from the tail (Duran et al., 2017). Studies in other species showed inconsistent results regarding the influence of body region on HCC (e.g., rabbits: Comin et al., 2012; coyote: Schell et al., 2017, sheep: Fürtbauer et al., 2019). Possible explanations for this inconsistency between animal species could be different hair growth cycles with diverse lengths of growth phases, species-specific shedding rhythms or diverging hair structures (Neurand et al., 1980; Meyer et al., 1980). Additionally, differences in HCCs between body regions of the same animal may be caused by an enhanced "wash out" of hair cortisol (e.g., by UV-radiation (Wester et al., 2016) and grooming (Acker et al., 2018)), increased skin blood flow (Carlitz et al., 2015) or elevated incorporation due to external contamination. Furthermore, body sites may differ in hair color (Sotohira et al., 2017) or hair type (Tallo-Parra et al., 2015), which may be additional influencing factors.

4.2. Age and sex

Our finding of elevated hair cortisol levels in piglets and calves after birth is in accordance with findings in other species (horses: Montillo et al., 2014; macaques: Grant et al., 2017). Indeed, hair collected at this time was grown *in utero* (Meyer and Görgen, 1986) and therefore may reflect enhanced prenatal cortisol concentrations during late gestation. As found in pigs, maternal cortisol levels increase throughout gestation (Otten et al., 2013) and fetal cortisol concentrations during late gestation (Kanitz et al., 2012), and plasma concentrations in piglets after birth are elevated compared to later ages (Otten et al., 2013). In

addition, young animals have lower corticosteroid binding globulin levels, resulting in higher unbound plasma cortisol concentrations (Kanitz et al., 2012; Grant et al., 2017), which may contribute to the increased HCCs at this age. There was a striking decrease in HCC from 2-week-old piglets to 10-week-old pigs in our study, which may be due to shedding of the birth coat (Slee, 1963; Ling, 1970). Furthermore, we found an increase in cortisol concentrations in sows compared to younger animals. Elevated HCCs at older ages were also described in humans and primates (Dettenborn et al., 2012; Fourie et al., 2015), where the increase was attributed to reduced HPA axis feedback sensitivity (Sapolsky et al., 1986). In our study, elevated HCCs in sows could also be caused by their reproductive cycle, as preliminary data indicate that hair samples collected in the insemination period exhibit higher HCC than samples taken in other periods of the reproductive cycle (unpublished data).

Our findings regarding the lack of sex differences in HCC are in accordance with previous reports in some species (Asiatic black bears: Malcolm et al., 2013; Canada lynx: Terwissen et al., 2013; pigs: Roelofs et al., 2018; dogs: Packer et al., 2019). However, several studies indicate higher HCC in males than in females (e.g., horses: Medill et al., 2015; chimpanzees: Yamanashi et al., 2016; muskoxen: Di Francesco et al., 2017) or elevated HCC in females compared to males (e.g., bears: Bechshoft et al., 2011; primates: Fourie et al., 2016; goats: Dulude-de Broin et al., 2019). These diverging findings may result from different habitats or housing conditions that may influence HPA axis activity. Free-ranging animals may also exhibit more distinctive sex-specific behavior, such as territorial fights and mating rituals, which may raise cortisol levels in males (Bergman et al., 2005). In contrast, sexual behavior is reduced in farm animals that are often housed in gender-separated groups or were sometimes castrated, as is the case in our study. Castration may lead to lower HCC, as shown in cats (Finkler and Terkel, 2010). The lack of differences in HCC between males and females in our study may be further caused by the rather limited sample from older animals. We were only able to sample newborn cattle and castrated male pigs until the age of 27 weeks because male pigs and cattle are almost exclusively intended for fattening and merely reach the age of sexual maturity.

4.3. Hair color

Our results regarding hair coloration and HCC are in line with findings from a previous study in cattle (Tallo-Parra et al., 2015). Comprehensive studies in humans provide further evidence that dark hair is related to higher HCCs (Rippe et al., 2016; Binz et al., 2018). The underlying mechanisms have not been elucidated, but it is speculated that an increased blood flow in skin covered by black hair may favor the incorporation of cortisol via blood vessels (Burnett et al., 2014) or that the number of melanocytes facilitates the incorporation of lipophilic substances (Pragst and Balikova, 2006). In addition, it is possible that melanin in black hair could have a UV filtering effect and thereby inhibit the degradation of cortisol by UV radiation. However, within the same animal, different-colored hair is often collected from different body regions, as is the case with our study in piglets. Due to the breed-specific markings of Saddleback pigs, white hair could only be shaved from the chest, whereas black hair was shaved from the back region. Therefore, the effect of hair color on HCC in piglets is not independent of the influence of body region. In contrast with our results, other studies reported higher HCCs in white hair compared to black hair in cattle (Burnett et al., 2014; González-de-la-Vara et al., 2011) and chimpanzees (Yamanashi et al., 2013).

4.4. Hair segment

In our study, the hair cortisol concentrations of hair cortisol in most distal segments were approximately 11 times higher than proximal segments in pigs and 2.5 times higher in cattle. To the best of our knowledge, an increase in HCC along the hair shaft was only reported in two previous studies on Asiatic black bears (Malcolm et al., 2013) and sheep (Fürtbauer et al., 2019). The majority of studies in other species revealed a decline in cortisol concentrations from proximal to distal hair segments, the so-called "washout effect" (e.g., humans: Kirschbaum et al., 2009; chimpanzees: Carlitz et al., 2015; cattle: Nedić et al., 2017). Studies in humans suggest that UV radiation, hair washing and hair styling can cause degradation or elimination of HCC (Hamel et al., 2011; Stalder et al., 2017; Wester et al., 2016; Grass et al., 2016). In addition, sunlight, hair washing or even natural friction can degrade hair cuticle cells (Dawber, 1996; Richena and Rezende, 2016). Since distal hair segments were exposed to external influences for a longer period of time, their surface structure may be more damaged. Our observation that hairs of the most distal hair segment in pigs often showed split ends may support this assumption. Thus, these parts of the hair may be more susceptible to external contamination by cortisol-containing fluids, e.g., sweat, sebum, saliva and urine, either from the animal itself or from conspecifics. In such cases, cortisol from external sources may not only accumulate at the hair surface but could also be incorporated into the hair shaft, causing an increase in HCCs. Further research should focus on the mechanisms of incorporation and elimination of hair cortisol for validation of HCC as a reliable stress indicator.

4.5. Seasonality

The results of our study show no differences in HCC between hair samples collected in summer or winter season in pigs; however, in cattle, increased HCCs were found in winter compared to summer samples. Previous studies also reported elevated HCCs during winter in cattle (Braun et al., 2017), pigs (Bacci et al., 2014) and muskoxen (Di Francesco et al., 2017), whereas in Japanese cattle, enhanced cortisol levels were found in samples shaved in June followed by a decrease until September and a renewed increase in samples taken in December (Uetake et al., 2018). Season is a complex influencing factor comprising numerous determinants, which may vary over the year, e.g., temperature, daylight, weather conditions or season-specific sexual, territorial or nutritional behavior. These factors and the use of different species and sampling times hamper the comparability of HCC results between studies. In addition, diverging cortisol incorporation in hair over a period of one year could be caused by seasonal changes in coat characteristics. In the northern hemisphere, winter hair of animals is commonly longer and denser than hair coat during the summer season (Berman and Volcani, 1961; Mowafy and Cassens, 1976) and can be accompanied by an enhanced growth of undercoat in some species, which seems to have lower HCCs than guard hair (Hayman and Nay, 1961; Macbeth et al., 2010; Dulude-de Broin et al., 2019).

The seasonal sampling times in our study were chosen to reflect periods around summer and winter solstice with a maximum difference in day length. The pigs were housed indoors only and thus likely less susceptible to external influences. However, cattle in our study were kept under freely ventilated loose housing conditions and may be more affected by changing environmental conditions, such as daylight, ambient temperature and precipitation. Previous studies indicate that sunlight and UV radiation degrade cortisol in hair (Wester et al., 2016). The greater exposure of cattle to these influences during summer months may therefore be a reason for lower HCCs. In addition, Sharma et al. (2019) found a positive correlation between HCC in the tail hair of cows and the occurrence of dirty flanks in the animals and dung in the lying area. Thus, seasonal differences in the degree of contamination in farm animals may also contribute to seasonal variations of HCCs.

5. Conclusions

Our findings demonstrate that age, body region, hair color, age of the hair segments and season are influencing factors on HCCs and have

similar effects in pigs and cattle. These factors should thus be considered and standardized whenever possible when using hair cortisol as a potential marker for increased stress in these species. For example, it is advisable to only compare HCC results from animals of the same age group with hair samples from the same body region and of the same hair color. Since our study revealed highly elevated cortisol levels in the hair of newborn animals with a subsequent decrease, we also recommend considering animals at older ages for comparison. Further, at least in cattle and possibly in other farmed animals that are housed partly outdoors, the season of sampling should be taken into account because HCCs are also affected by seasonal influences.

In addition, the age of hair segments seems to be a confounding factor that cannot be controlled or standardized as easily as the other influencing factors. Considering the remarkable increase of HCC from proximal to distal hair segments in our study, it is advisable to use regrown hair from previously shaved areas for the reflection of a particular time period. This approach will help to minimize the confounding effect of cortisol accumulation in older hair segments, which may be caused by contamination. For the use of HCC as an indicator of stress and long-term activation of the HPA axis, it is therefore advisable to use hair sampling protocols with the best possible standardization of the identified influencing factors. In addition, the mechanisms of incorporation and accumulation of cortisol into the hair shaft should be further investigated.

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CRediT authorship contribution statement

Susen Heimbürge: Conceptualization, Methodology, Investigation, Writing - original draft. **Ellen Kanitz:** Conceptualization, Methodology, Writing - review & editing, Funding acquisition. **Armin Tuchscherer:** Formal analysis. **Winfried Otten:** Conceptualization, Methodology, Investigation, Writing - review & editing, Funding acquisition.

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4.3 Study 3: Effects of long-term stress on hair cortisol concentrations

Is it getting in the hair? Cortisol concentrations in native, regrown and segmented hairs of cattle and pigs after repeated ACTH administrations

Susen Heimbürge, Ellen Kanitz, Armin Tuchscherer, Winfried Otten

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Highlights:

- Repeated ACTH applications increase hair cortisol levels (HCC) in cattle and pigs.
- In cattle, HCCs in natural, regrown and segmental hair reflect HPA axis activity.
- Pigs show a blunted cortisol response and no differences in HCCs between groups.
- Seasonal hair growth and contamination may also mask treatment effects in pigs.

Statement of contribution:

I contributed to the third study by designing and conducting the experiments and analysing the sampling material with the assistance of technicians at the Institute of Behavioural Physiology. Additionally, I gathered and analysed the data, interpreted the results and prepared the manuscript and figures, which were edited by the co-authors before submission.

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Research paper

Is it getting in the hair? – Cortisol concentrations in native, regrown and segmented hairs of cattle and pigs after repeated ACTH administrations

Susen Heimbürge[a], Ellen Kanitz[a], Armin Tuchscherer[b], Winfried Otten[a,*]

[a] Leibniz Institute for Farm Animal Biology (FBN), Institute of Behavioural Physiology, Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany
[b] Leibniz Institute for Farm Animal Biology (FBN), Institute of Genetics and Biometry, Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany

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ABSTRACT

Adrenocorticotropic hormone (ACTH) is part of the hypothalamic-pituitary-adrenal axis response to stress and induces the release of cortisol, which is commonly used as an indicator in stress and animal welfare research. In recent years, hair cortisol concentration (HCC) gained increasing importance as a promising retrospective indicator for stress in animals. Thus, the aim of our study was to validate HCC as a potential indicator of increased endogenous cortisol release in cattle and pigs by repeated ACTH administrations followed by cortisol analysis in different hair types. For this purpose, 34 cattle and 38 gilts were treated either with repeated i.m. injections of ACTH or saline every second day over a period of 4 weeks. Saliva samples were taken before and after injections once a week from selected animals to verify the endogenous cortisol response. At the end of the treatment (week 4) and after 8 and 12 weeks, samples of natural and regrown hair were taken from the caudo-dorsal region of the back and analyzed for cortisol concentrations. In addition, natural hair was sampled after 12 weeks and cut into segments prior to analysis. Treatment with ACTH revealed a significant increase in salivary cortisol after application in both species, although this increase was attenuated in pigs compared to cattle. In week 4, HCCs were significantly elevated in natural and regrown hair of ACTH-treated animals. In cattle, HCCs significantly increased after ACTH treatment in natural, regrown and segmental hair compared with control animals, indicating that HCC may be a promising indicator of stress, as cortisol levels in all hair types reflected the preceding period with increased cortisol release. In pigs, there were no differences in HCCs between treatments. This may be caused by the lower systemic cortisol response in pigs, but seasonally reduced hair growth and external cross-contamination of hair by saliva and urine under commercial husbandry conditions may also interfere with the validity of HCC in this species.

1. Introduction

Under commercial husbandry conditions, farm animals may suffer from diverse stressful situations, such as poor housing conditions, social stress and frequent management procedures (Wood-Gush et al., 1975). Thus, there is growing interest in the use of animal-based, minimally- or non-invasive stress indicators to assess wellbeing in farm animals (Palme, 2012; Barrell, 2019). In addition to behavioral parameters and physical indicators, the analysis of hormones, such as cortisol, is commonly used for the assessment of stress and animal welfare (Mormède et al., 2007). Since the measurement of cortisol in blood, saliva, urine and feces provides several disadvantages, such as frequent and invasive sampling and circadian variation, hair is increasingly used as a promising alternative sampling material (Heimbürge et al., 2019). Numerous studies have already shown that diverse stressors such as crowding (Schubach et al., 2017), lower social rank (Qin et al., 2013) and the occurrence of diseases (Comin et al., 2013) are reflected in elevated hair cortisol concentrations (HCCs). However, further validation of the suitability of HCC as an indicator of long-term stress is needed to investigate the relationship between HCCs and systemic cortisol levels in farm animals. To this end, administrations of adrenocorticotropic hormone (ACTH) are used to simulate stress-induced release of endogenous cortisol from the adrenal glands (Kersey and Dehnhard, 2014). Since it is assumed that the main source of hair cortisol is circulating, unbound cortisol, which diffuses from the blood vessels into the growing hair (Stalder and Kirschbaum, 2012), an increased incorporation of cortisol into the hair shaft should be detectable after ACTH administrations, as already shown in previous studies (del González-de-la-Vara et al., 2011; Dulude-de Broin et al., 2019). Thereby, repeated ACTH administrations can be used for the validation

* Corresponding author.
E-mail address: otten@fbn-dummerstorf.de (W. Otten).

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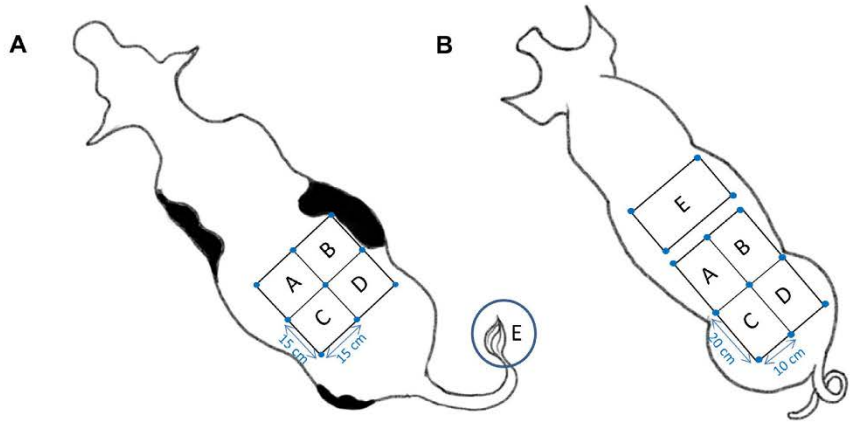

Fig. 1. Areas for hair sampling in cattle (A) and pigs (B).

of HCC as a retrospective indicator of preceding periods with increased hypothalamic–pituitary-adrenal (HPA) axis activity.

In this context, the aim of our study was to determine the relationship between repeated ACTH applications and hair cortisol concentrations in two different species (cattle and pigs), at different sampling times and for the first time in three different hair sample types (natural, regrown and segmented hair). During a 4-week treatment period either with i.m. injections of ACTH or saline, saliva samples were collected before and after administrations to verify the increased endogenous cortisol response. Natural and regrown hair samples were taken at three sampling times after the treatment period. Furthermore, hair segments supposed to represent different time periods were collected at the end of the experiment. Based on average hair growth rates in cattle and pigs, we set up models and then tested whether and after which time period prolonged increased systemic cortisol levels are reflected by increased HCC in different hair sample types of cattle and pigs.

2. Materials and methods

All procedures performed in this study involving animal handling and treatments were in accordance with the German animal protection law and were approved by the local ethics committee (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei, Mecklenburg-Vorpommern, Germany; LALLF M − V/AZ 7221.3–1.1–059/17).

2.1. Animals and housing

In cattle, the study population consisted of 34 female Holstein Friesian cattle with a mean ± SD age at the start of the experiment of 7.9 ± 0.5 months and a body weight of 240.6 ± 41.5 kg. Cattle were housed in the Experimental Cattle Facility of the Leibniz Institute for Farm Animal Biology (FBN Dummerstorf) and were fed and watered ad libitum. Two replicates were carried out between September and January with 18 and 16 animals, respectively. Within each replicate, animals were group-housed on slatted floors (83 m^2) with free stalls.

A further experiment was performed using 38 German Landrace gilts with a mean ± SD age of 4.5 ± 0.5 months and a body weight of 90.3 ± 10.6 kg at the start of the experiment. An additional four animals served as reserves to standardize the group size. All gilts were bred and raised in the Experimental Pig Facility of the FBN Dummerstorf. Animals were fed a commercial diet according to the recommendations for their age. Feeding times were twice daily at 7:30 a.m. and 2:00 p.m. and water was available ad libitum. Gilts were housed in groups of seven animals per pen (slatted floor, 9 m^2), and a total of three replicates with two animal groups each were carried out between March and August.

2.2. Experimental design

A consistent experimental protocol was used in both cattle and pigs. Two weeks before starting the experiments, all animals were allowed to adapt to the housing conditions and were habituated to the handling procedures. Within each replicate, half of the cattle or gilts were randomly allocated to receive ACTH or control treatments. Thus, all animal groups consisted of both ACTH and control animals. The treatment of ACTH animals involved repeated administrations of 2 ml ACTH solution (100 IU Synacthen Depot, Alfasigma S.p.A., Milano, Italy). The dose was chosen based on dose–response curves and results already described in previous studies using this ACTH dose in cattle (Dobson et al., 2000; Biran et al., 2015) and pigs (Otten et al., 2004; Backus et al., 2013). Control animals received repeated injections of 2 ml saline. All animals were injected intramuscularly in the neck region around 9:00 a.m. every second day over a period of 4 weeks, resulting in a total of 14 injections per animal. After the 4-week treatment period, animal groups were kept for an additional eight weeks for hair sampling.

Hair samples were taken from the caudo-dorsal region of the back one day before the start of the treatment period (0), after four weeks (end of treatment) and after eight and 12 weeks. Therefore, the caudo-dorsal region was divided in four areas (Fig. 1). At the beginning of the experiment, a first sample of natural hair was taken in area “A” by shaving the hair as close to the skin as possible using an electric clipper (Wahl Professional KM 10, Mosonmagyaróvár, Hungary) with a special trimmer blade (Oster Cryogen-X Nr. 50 (0.2 mm), Boca Raton, USA). After 4 weeks, natural hair in area “B” and regrown hair in area “A” was shaved. Accordingly, after 8 and 12 weeks, natural hair was shaved in areas “C” and “D” respectively, and samples of hair regrown during the preceding four weeks were taken from previously shaved areas. There were no differences in hair color within and between pigs. In cattle, the predominant hair color in the caudo-dorsal region (black or white) of each animal determined the color of all hair samples of that animal.

After 12 weeks, additional samples of natural hair were taken from the tail tip of cattle and the back region of pigs to investigate the cortisol concentrations in three hair segments (area “E”). Considering the different hair growth rates in these regions of cattle and pigs a total hair length of 4.5 cm in cattle and 3.0 cm in pigs was chosen to represent the entire 12-week period of the experiment (Heimbürge et al., 2020).

Thus, in cattle, proximal hair strands of the tail tip were collected and subsequently cut in three 1.5-cm segments (area "E", Fig. 1 A). In pigs, hairs were directly harvested in three 1-cm segments in area "E" (Fig. 1 B), by using an adjustable electric clipper (hair clipper series 1000 QC5005/10, Philips, Drachten, Netherlands). Hair samples were collected by hand or, in case of regrown hair and hair segments in pigs, by using a vacuum cleaner (cleanmaxx zyklon PC-P006EA, DS Produkte GmbH, Gallin, Germany) with a paper filter in the suction tube. All hair samples were dried, weighed and stored light-protected at room temperature (RT) until analysis.

To verify the endogenous cortisol release by ACTH during the treatment period, saliva samples were taken from 20 cattle (ACTH and control: n = 10, respectively) as well as from 24 gilts (ACTH and control: n = 12, respectively) for analysis of salivary cortisol concentrations. Saliva sampling was repeatedly performed on one day per week during the 4-week treatment period. Starting at 8:30 a.m. and 30 min before the ACTH/saline application, the first saliva sample was collected by allowing the animals to chew on veterinary cotton swabs until they were thoroughly moistened. Additional samples were taken 1, 2, 3, 6 and 9 h after application. The cotton swabs were placed in tubes, centrifuged at 2,500 g for 10 min at 4 °C and stored at −20 °C until analysis.

2.3. Analysis of cortisol

After thawing, saliva samples were spun at 2,500 g for 5 min, resulting in a clear supernatant with low viscosity. The analysis of cortisol concentrations in 50 µl saliva samples was performed in duplicate using an ELISA kit for salivary cortisol (Demeditec Diagnostics GmbH, Kiel, Germany) as described by Goursot et al. (2019). For cattle, the sensitivity of this assay was 0.06 ng/ml and the intra- and inter-assay coefficients of variation (CV) were 4.2% and 8.6%, respectively. For pigs, sensitivity of the assay was 0.08 ng/ml and the intra- and inter-assay CV were 3.4% and 7.2%, respectively.

Extraction and analysis of hair cortisol was performed using the procedures described by Heimbürge et al. (2020). Briefly, hair samples were washed twice with isopropanol dried at RT, snap-frozen in liquid nitrogen and pulverized using a ball mill (MM 400, Retsch GmbH, Haan, Germany). For cortisol extraction, 1 ml of methanol (HPLC grade) was added to approximately 50 mg of pulverized hair and incubated at RT for 18–24 h with slow shaking. Then, the samples were spun in a microcentrifuge at 12,000 rpm for 2 min, and 0.6 ml of the supernatants were finally dried using a vacuum centrifuge (SpeedVac Concentrator, Thermo Fisher Scientific Inc., Waltham, MA, USA) and stored at −20 °C. The dried extracts were reconstituted in 0.4 ml phosphate buffer and analyzed for hair cortisol using the same ELISA kit as for salivary cortisol (Demeditec Diagnostics GmbH, Kiel, Germany). The cross-reactivities of the antibody with other steroids were as follows: testosterone, < 0.1%; corticosterone, 6.2%; cortisone, 0.8%; 11-deoxycorticosterone, 2.6%; 11-deoxycortisol, 50%; dexamethasone, < 0.1%; estriol, < 0.1%; estrone, < 0.1%; prednisolone, 100%; prednisone, 0.9%; progesterone, < 0.1%; 17-hydroxyprogesterone, 1.3%; danazole, < 0.1%; pregnenolone, < 0.1%; estradiol, < 0.1%; androstenedione, < 0.1%. The sensitivity of the assay was 0.8 pg/mg for cattle hair and 1.1 pg/mg for pig hair. Serial dilutions of hair extracts showed parallelism to the cortisol standard curve with no significant difference in slope ($p > 0.05$). The intra- and inter-assay CV were 4.3% and 7.8% for cortisol analyses in hair of cattle, and 4.5% and 6.8% for hair cortisol in pigs, respectively.

2.4. Statistical analyses

Statistical analyses were conducted with the SAS System for Windows, version 9.4 (Copyright, SAS Institute Inc., Cary, NC, USA). All data were analyzed using the MIXED procedure for performing analyses of variance (ANOVA) with hair cortisol or saliva cortisol concentrations as the dependent variables. Data from natural and regrown hair samples from cattle and pigs were analyzed with a model comprising the fixed factors treatment (ACTH, control) and replicate, the repeated factor sampling time (0, 4, 8, 12 weeks) and the interaction of treatment and sampling time. For the statistical evaluation of the hair segments in both species, data were analyzed using a model considering the fixed factors treatment and replicate, the repeated factor segment (1–3) and the interaction of treatment and segment. In cattle, the additional factor hair color was included in the models for HCC in natural and regrown hair, and in pigs, the additional factor animal group (nested within replicate) was included in all models. Furthermore, HCCs were compared between natural und regrown hair of control animals using a model which included the fixed factor hair type (natural, regrown), the repeated factor sampling time (4, 8, 12 weeks) and the interaction of these factors. The statistical model for saliva cortisol in cattle and pigs comprised the fixed factors treatment and replicate, the repeated factors sampling time (-0.5, 1, 2, 3, 6, 9 h) and treatment week (1–4) and all two-way interactions with treatment. AUC (area under curve) was calculated from the saliva cortisol responses of 10 cattle and 12 pigs treated with ACTH, and Pearson correlation coefficients between AUC and the HCCs after the treatment period were estimated with the CORR procedure to evaluate their relationship. Repeated samplings on the same animal were taken into account by the repeated statement of the MIXED procedure using a compound symmetry structure of the block diagonal residual covariance matrix. Multiple pairwise comparisons were made using the Tukey-Kramer test, and the SLICE statement of the MIXED procedure was used to perform partitioned analyses of the least squares means (LSM) for all interactions. Significance was considered at $p < 0.05$. All results are expressed as LSM ± standard error (SE).

3. Results

3.1. Salivary cortisol

The results of the ANOVA revealed significant effects of treatment (both species: $p < 0.001$), sampling time (both species: $p < 0.001$) and treatment × sampling time (cattle: $p < 0.001$, pigs: $p < 0.01$) on cortisol concentrations in saliva. In cattle, pairwise comparisons indicated significantly increased cortisol concentrations in ACTH-treated animals compared with controls at all sampling times after injection (Fig. 2 A). In pigs, salivary cortisol was significantly elevated in ACTH compared with control animals 1, 2 and 3 h after application (Fig. 2 B). The highest saliva cortisol concentrations were found 2 h (cattle) and 3 h (pigs) after ACTH application.

3.2. Hair cortisol

Cortisol concentrations in natural and regrown hair samples of cattle and pigs are shown in Fig. 3. In cattle, ANOVA revealed significant effects of treatment, sampling time, and their interaction on cortisol concentration in natural hair (all $p < 0.001$). Pairwise comparison showed elevated HCCs in ACTH compared with control animals in weeks 4, 8 and 12 (all $p < 0.001$). Additionally, cortisol was significantly higher in weeks 4, 8 and 12 than in week 0 (all $p < 0.001$; Fig. 3 A). Furthermore, hair color revealed significant effects on cortisol in natural hair ($p < 0.001$). Thus, cortisol concentrations in black hair were significantly higher compared with white hair (black: 19.6 ± 1.4 pg/mg, white: 18.2 ± 1.4 pg/mg). In regrown hair samples of cattle, the results of the ANOVA indicated a significant influence of treatment, sampling time and the interaction of treatment and sampling time on cortisol concentrations (all $p < 0.001$, Fig. 3B). The pairwise comparison revealed increased HCCs in samples from ACTH-treated animals compared with control animals in week 4 ($p < 0.001$) and week 8 ($p < 0.01$). Elevated cortisol concentrations were also found in regrown hair in week 4 ($p < 0.001$) and 8 ($p < 0.01$) in comparison to week 12 in the ACTH animals. Hair color

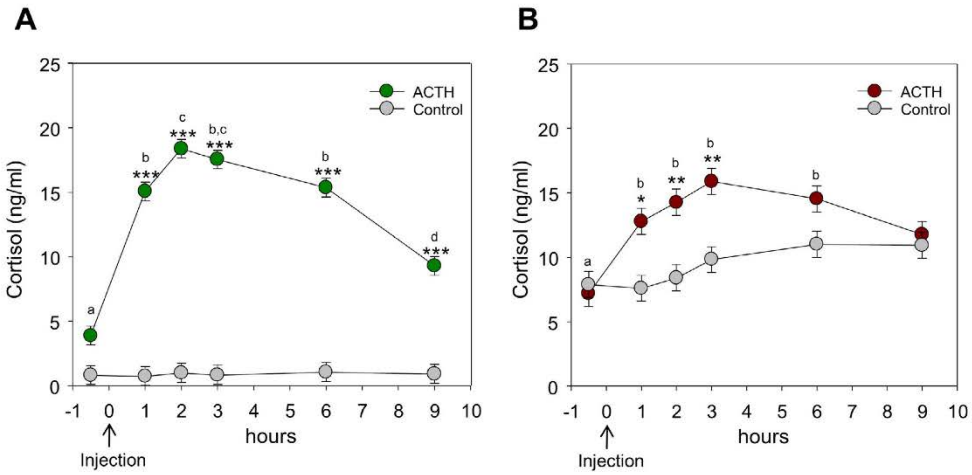

Fig. 2. Salivary cortisol concentrations after ACTH/saline injection in cattle (A) and pigs (B). Data are presented as LSM ± SE, and significant differences between treatment groups within sampling times are indicated by asterisks (***p < 0.001, **p < 0.01, *p < 0.05). Significant differences between sampling times within treatment groups are indicated by different letters ($^{a, b, c, d}$p < 0.05).

had no significant effect on cortisol in regrown hair (black: 27.5 ± 4.6 pg/mg, white: 26.6 ± 4.8 pg/mg).

In pigs, cortisol levels in natural hair samples did not differ between ACTH-treated and control animals (p = 0.10), however, ANOVA revealed a significant effect of sampling time and animal group on HCCs (both: p < 0.001). Pairwise comparisons indicated a significant

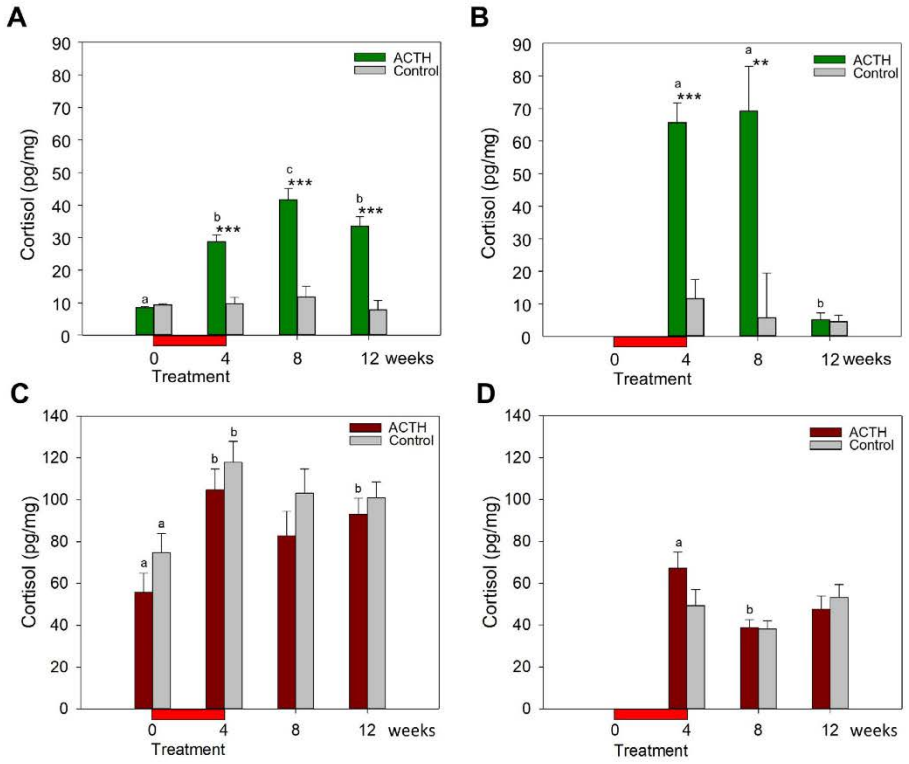

Fig. 3. (A-B) Cortisol concentrations in natural (A) and regrown (B) hair samples in cattle. (C–D) Cortisol concentrations in natural (C) and regrown (D) hair samples in pigs. Data are presented as LSM ± SE, and significant differences between treatment groups within sampling times are indicated by asterisks (***p < 0.001, **p < 0.01). Significant differences between sampling times within treatment groups are indicated by different letters ($^{a, b, c}$p < 0.05).

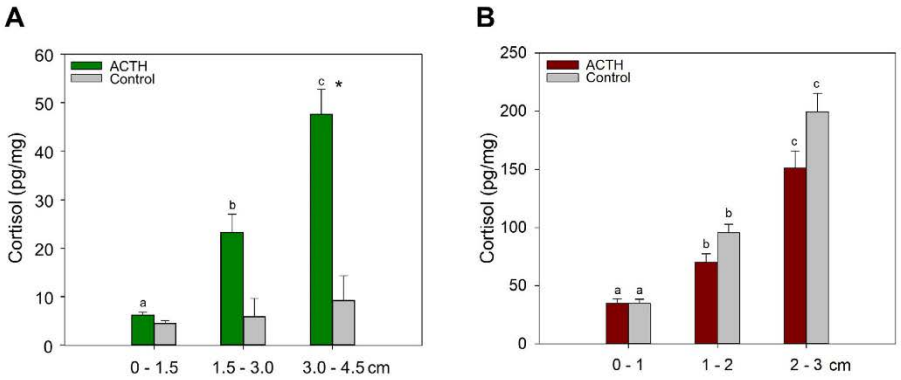

Fig. 4. Cortisol concentrations in hair segments of cattle (A) and pigs (B). Data are presented as LSM ± SE, and significant differences between treatment groups within sampling times are indicated by asterisk (*$p < 0.05$). Significant differences between sampling times within treatment groups are indicated by different letters ($^{a, b, c}p < 0.05$).

increase in HCC in samples shaved in week 4 in comparison with samples shaved before the treatment period (week 0) in animals of both treatments (all $p < 0.001$; Fig. 3 C). In addition, HCCs in ACTH animals were significantly elevated in week 12 compared with week 0. In regrown hair samples of pigs, ANOVA revealed a significant effect of sampling time ($p < 0.001$) and animal group ($p < 0.01$) on HCC, but no significant effect of treatment or the interaction of treatment and sampling time. Pairwise comparisons showed significantly elevated HCCs in regrown hair of ACTH-treated pigs in week 4 compared to week 8 ($p < 0.01$; Fig. 3 D).

In hair segments, ANOVA indicated a significant influence of treatment on HCCs (cattle $p < 0.001$, pigs: $p < 0.05$). Furthermore, the hair segment (cattle: $p < 0.01$, pigs: $p < 0.001$) and the interaction of segment and treatment (cattle: $p < 0.01$, pigs: $p < 0.05$) also had significant effects on HCCs in both species. Pairwise comparisons revealed higher HCCs in ACTH-treated cattle compared with controls ($p < 0.001$), and cortisol concentrations increased from the proximal to the distal hair segments in ACTH-treated animals (Fig. 4 A). In contrast, ANOVA showed generally higher HCC in control than in ACTH pigs ($p < 0.05$). Hair cortisol levels increased from proximal to distal hair segments in ACTH and control pigs (Fig. 4 B).

In control cattle and pigs there were significant differences between cortisol levels in natural and regrown hair. Cortisol concentrations were higher in natural compared with regrown hair in both species (cattle: 9.8 ± 0.5 pg/mg vs. 7.4 ± 0.5 pg/mg, $p < 0.01$; pigs: 108.6 ± 7.5 pg/mg vs. 48.6 ± 7.5 pg/mg, $p < 0.001$).

The statistical analysis of the relationship between systemic cortisol release and HCC in cattle revealed a significant correlation of the AUC of the saliva samples with HCC in natural hair collected in week 8 ($r = 0.84$, $p < 0.01$) and week 12 ($r = 0.93$, $p < 0.001$), and with HCC in regrown hair samples from week 4 ($r = 0.95$, $p < 0.001$). In pigs, there were no significant correlations between saliva and hair cortisol.

4. Discussion

To the authors' knowledge, this is the first study in which three different hair sample types (natural, regrown and segments) were taken from animals to determine whether and at which sampling time repeated ACTH applications lead to an increase in hair cortisol concentrations.

4.1. Saliva

Numerous studies revealed a high correlation between cortisol concentrations in saliva and cortisol levels in blood in various species (Dorn et al., 2007; Hellhammer et al., 2009; Negrao et al., 2004; Hernandez et al., 2014; Cook et al., 1996), indicating that salivary cortisol can reliably reflect changes in systemic cortisol concentrations. Based on our results from saliva samples we could show that the application of ACTH increased systemic cortisol levels for several hours and resulted in repeated activation of the HPA axis over a period of 4 weeks in cattle and pigs. Thus, our model proved to be effective in simulating the cortisol response to a chronic intermittent stressor. However, comparing both species, the salivary cortisol response was more attenuated in pigs than in cattle with significantly increased cortisol levels for between 3 and 6 h in pigs compared with 9 h in cattle after ACTH application. In comparison, the repeated applications of saline did not alter salivary cortisol levels in control animals, indicating no effect of the treatment itself.

4.2. Natural hair

The hair follicle is located approximately 2 mm under the skin surface in cattle (Udo, 1978) and 3.5 mm in pigs (personal data). Hair in the back region grows approximately 3.5 mm per month in cattle and 7 mm per month in pigs (Heimbürge et al., 2020). Based on these data, we created a model for hair growth in pigs and cattle and hypothesized that natural and actively growing hair of ACTH animals should exhibit increased HCC in week 4 and a maximum concentration in week 8, when it should contain the total amount of cortisol incorporated during the treatment period. We further predicted a decrease in HCC four weeks later (week 12), because newly growing hair portions with basal cortisol levels may lead to a dilution effect (Fig. 5). Due to the cyclic and season-dependent hair growth, samples of natural hair comprise different amount of hairs being in the anagen, catagen and telogen phase. Since only actively growing hair in the anagen phase incorporates cortisol from the blood stream, hair in other stages of the growth cycle could also cause a dilution effect on HCCs in natural hair.

In our study, results in cattle reveal a pronounced increase in cortisol concentrations in all natural hair samples of ACTH-treated animals after the treatment period, with highest cortisol levels in week 8 (4 weeks after the end of treatment), supporting our hypothesis. Furthermore, saliva samples that were collected during the 4-week treatment period and therefore show elevated cortisol levels in ACTH animals, best correlated with samples harvested in week 8 and 12,

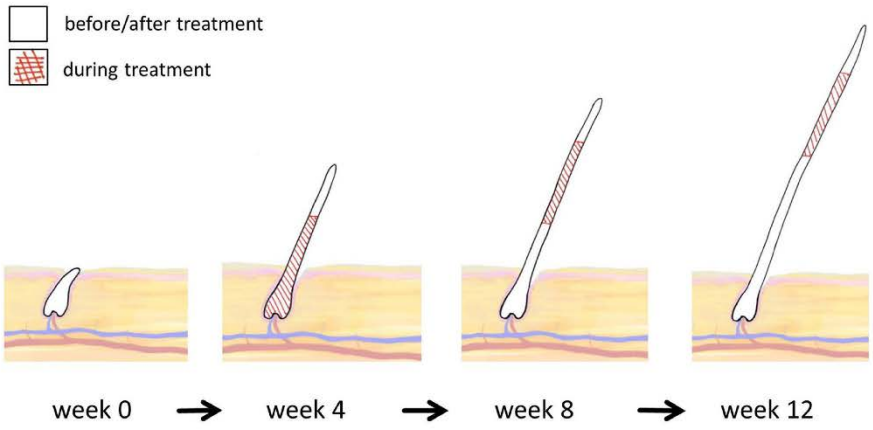

Fig. 5. Hair growth model of natural, unshaved hair in the anagen phase during the 12-week experimental period in cattle and pigs. Shading illustrates hair sections with increased cortisol incorporation during the 4-week treatment period.

because at this time all of the increased cortisol was located in the hair shaft over the skin. Additionally, cortisol concentrations of natural hair samples from control animals remained unaffected independent of the week of sampling. Interestingly, natural black hair showed significantly higher HCC than natural white hair, although this effect was not significant in regrown hair samples. Higher HCC in black hair was also reported in cattle and humans (Tallo-Parra et al., 2015; Heimbürge et al., 2020; Binz et al., 2018), whereas other studies showed contradictory results (Burnett et al., 2014, Yamanashi et al., 2013). It is discussed that melanin or an improved blood supply of skin covered with black hair facilitates the incorporation of cortisol (Pragst and Balikova, 2006; Burnett et al., 2014). A further explanation could be a possible degradation of cortisol caused by UV radiation, which might be stronger in white hair because in black hair the pigment may have a sun protective function. Newly grown hair was not as long under UV radiation as natural hair so this effect may not be detectable in regrown hair samples. Our findings regarding the effect of ACTH application on HCC are in accordance with results of a previous study in dairy cattle, where animals received either ACTH or saline weekly for 3 weeks (del González-de-la-Vara et al., 2011). The authors found elevated HCCs in samples from day 14 (directly at the end of treatment) and day 28 (14 days after treatment period) in ACTH-treated cattle, whereas saline-injected and untreated animals showed no increase in HCC.

In contrast, in pigs, we found enhanced cortisol concentrations in natural hair in week 4 (end of the treatment) compared with basal values in week 0. However, no significant differences were found between ACTH and control animals at all sampling times. The lack of differences in HCC between treatments may be partly explained by the weaker salivary cortisol response following ACTH administration in pigs. A further explanation for the missing differences in porcine hair could be the annually hair growth pattern. As mentioned above, hair growth occurs in cycles of three growth phases, whereby cortisol is only incorporated in the anagen phase. In pigs, there is a pronounced seasonality of the follicle activity and porcine hair follicles pass through one cycle per year (Mowafy and Cassens, 1976; Watson and Moore, 1990). The hair growth during the anagen phase takes several months, predominantly during fall and winter. Thus, in our study, natural hair shaved in spring and summer may be mainly in catagen or telogen phases and therefore may incorporate less systemic cortisol.

Unexpectedly, cortisol levels were raised not only in hair samples of ACTH-treated gilts, but also in controls. However, there was no increase in systemic cortisol concentrations in control animals, as shown by salivary cortisol. Since animals in our study were housed under conventional conditions on slatted floors and in mixed groups consisting of ACTH and control animals, it is likely that hair samples from control animals were contaminated with urine and saliva from ACTH-treated animals, which contain high concentrations of cortisol. Contamination may be caused by lying on soiled floor or by social interactions, which frequently occur in group housing under restricted conditions with limited space. In line with this assumption, we could already show in an *in vitro* study with hair from pigs and cattle that HCC can be increased by contamination with body fluids such as urine (Otten et al., 2020). Additionally, Macbeth (2013) could show that contamination of culvert traps with urine and feces increased HCCs in brown bears. Thus, cross-contamination with saliva, urine and feces may lead to incorporation of external cortisol into the hair shaft and may thus compromise the usefulness of HCC as a stress indicator. In cattle, no effect of contamination was apparent. This may be because cattle were kept on a larger space with free stalls for lying and thus a presumably lower occurrence of soiling and social interactions may have occurred. Furthermore, cattle and pigs differ in their coat and hair characteristics and cattle hair may be less susceptible to contamination due to its density.

4.3. Regrown hair

For sampling of regrown hair, we applied the shave-reshave procedure every four weeks (Meyer and Novak, 2012). Thus, these samples contained only hairs that were actively grown within the preceding four weeks, and therefore might better reflect the systemic cortisol concentration during the previous weeks than natural hair. Assuming the hair growth rate and the follicle depth as described above, we expected increased and similar HCCs in samples shaved from ACTH animals in week 4 and week 8 (Fig. 6). In week 12, all hair portions with elevated HCC should have already been shaved off and no differences were expected (Fig. 6).

Results in cattle revealed high cortisol concentrations in regrown hair samples of ACTH-treated animals in week 4 and week 8 in comparison to control animals, which is in line with this hypothesis. In addition, the correlation of cortisol concentrations in saliva and regrown hair samples was highest in week 4, indicating the close relation between systemic cortisol and the cortisol level in this hair sample. No differences in HCCs between ACTH-treated and control animals were found in week 12. In pigs, HCC in regrown hair of ACTH animals was highest in week 4, and decreased until week 8. Similar to the results found in natural hair, there were no differences between ACTH and control pigs. Again, cross-contamination by body fluids and the attenuated cortisol response to ACTH in pigs compared with cattle may have affected the results for HCC in this hair type.

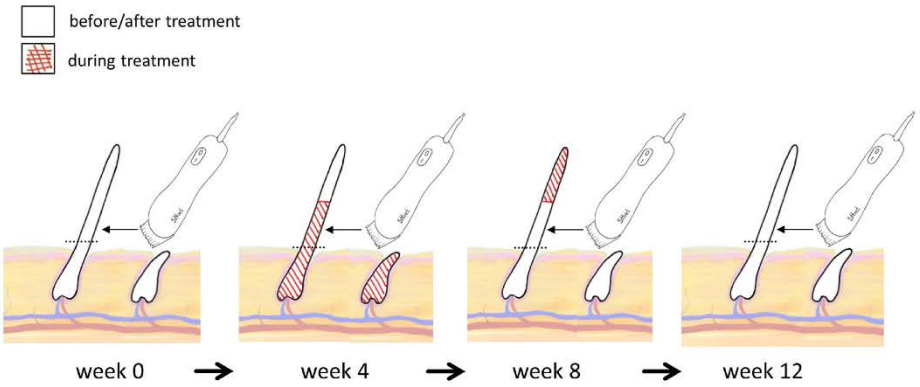

Fig. 6. Hair growth model of regrown hair in the anagen phase during the 12-week experimental period in cattle and pigs. Shading illustrates hair sections with increased cortisol incorporation during the 4-week treatment period.

Studies using ACTH administrations indicate that the number of applications and the duration of the treatment determine whether the cortisol response to ACTH is reflected in HCC. Studies in caribou, prairie dogs and bull calves where ACTH was injected only once or twice did not find any elevations in hair cortisol concentrations (Ashley et al., 2011; Crill et al., 2019; Tallo-Parra et al., 2017). Furthermore, daily treatment with ACTH for 7 days also had no effect on HCC in goats (Endo et al., 2018). In contrast, in a second experiment from the same study, goats were treated with ACTH twice daily over 2 weeks, and an increase in HCC was then detected. In line with our results, elevated HCCs were best found within one month after treatment but not in later samples, as shown in goats and cattle (Endo et al., 2018; del González-de-la-Vara et al., 2011). Thus, it can be assumed that periods with increased HPA axis activity should be at least two weeks long to be reflected in increased HCCs. Additionally, hair samples collected within four weeks after the period of interest best represent the treatment or stress load. Considering our hypothesis for regrown hair, it could be assumed that highest HCC may be found in hair samples collected two weeks after the end of treatment. However, an exact explanation of when elevated cortisol concentrations appear in the hair shaft would require an experiment with radiolabeled cortisol similar to those of Keckeis et al. (2012).

4.4. Hair segments

Studies in humans showed that hair segmentation can be applied to create a retrospective calendar of previous alterations in systemic cortisol levels over weeks or months (Kirschbaum et al., 2009). In our study in cattle and pigs, the length of hair segments was chosen to approximately reflect the hair growth during the 12-week experimental period within three segments. Since hair segments were shaved in week 12, HCCs might be elevated in the most distal segments of ACTH-treated animals. Supporting this assumption, we found a significant increase in HCC from the proximal to the distal hair segment in ACTH-treated cattle, while cortisol concentrations in all three segments of control animals remained unchanged. In pigs, and similar to the results in natural and regrown hair, HCC increased from proximal to distal segments not only in ACTH-treated but also in control animals. A possible explanation for this increase by both treatments could be the potential contamination effect as described above. The accumulation of external cortisol seems to be enhanced in older hair segments since these are exposed to external influences for longer and may exhibit more structural damage, e.g., by sunlight exposure or natural friction of hair cuticle cells (Dawber, 1996; Richena and Rezende, 2016; Grass 2017). This assumption is supported by our results showing that HCCs in younger regrown hair samples were significantly lower than in samples of older natural hair. Thus, in pigs, the effect of ACTH treatment could not be clearly separated from possible contamination and accumulation effects. In contrast to studies in e.g., humans (Kirschbaum et al., 2009), primates (Carlitz et al., 2015) and horses (Duran et al., 2017), where a wash-out effect of cortisol was observed from proximal to distal parts of the hair shaft, studies in sheep (Fürtbauer et al., 2019), bears (Malcolm et al., 2013), cattle and pigs (Heimbürge et al., 2020) reported elevated cortisol levels in distal hair segments. So far, only one study in bears could relate HCC in two different hair segments to preceding stressful events (Malcolm et al., 2013). Thus, our study is the first that applied cortisol concentrations in hair segments of farm animals as a possible retrospective calendar of preceding stress periods with elevated systemic cortisol levels. However, our results show that segmental analysis of HCC could reliably reflect previously increased HPA axis activity only in cattle.

5. Conclusion

The present study reveals that repeatedly elevated systemic cortisol concentrations over 4 weeks increase hair cortisol levels in both cattle and pigs. However, there was no difference in hair cortisol levels between ACTH-treated and control pigs. This may be due to the attenuated systemic cortisol response, a seasonally reduced hair growth and cross-contamination by urine and saliva. Thus, the prevention of these interfering factors seems to be a prerequisite for a possible applicability of HCC as an indicator of stress in pigs. In cattle, HCCs in natural, regrown and segmental hair samples reliably reflected the preceding period with increased systemic cortisol concentrations. Therefore, these three hair sample types can be used as a stress indicator in cattle. The most appropriate time for hair sampling seems to be the period within four weeks after the end of the stress period. Additionally, although there seems to be less interference due to contamination in cattle, it is recommended to perform the shave-reshave procedure and use regrown hair samples for analyses. Such hairs are exposed to external influences for a shorter time, contain more actively growing hairs and thus seem to have a higher retrospective validity than natural hair samples. Further research should focus on the investigation of confounding factors like cortisol-containing body fluids (e.g., saliva and urine) on HCC and on determination of mechanisms for incorporation of external cortisol into the hair shaft.

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CRediT authorship contribution statement

Susen Heimbürge: Conceptualization, Methodology, Investigation, Writing - original draft. **Ellen Kanitz:** Conceptualization, Methodology, Writing - review & editing, Funding acquisition. **Armin Tuchscherer:** Formal analysis. **Winfried Otten:** Conceptualization, Methodology, Investigation, Writing - review & editing, Funding acquisition.

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4.4 Study 4: Effects of contamination and elimination on hair cortisol concentrations

It's getting hairy – External contamination may affect the validity of hair cortisol as an indicator of stress in pigs and cattle

Winfried Otten, Susen Heimbürge, Ellen Kanitz, Armin Tuchscherer

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Highlights:

- External factors can influence hair cortisol concentrations (HCCs).
- Contamination is a confounding factor on HCC in pigs and cattle.
- Cortisol in urine causes a concentration-dependent increase in HCC.
- Repeated treatment with water leads to washout of cortisol from porcine hair.

Statement of contribution:

My contribution to the fourth study of my thesis comprised the conceptualisation, practical experimental work and analyses of the sampling material with the support of technicians at the Institute of Behavioural Physiology. In collaboration with the co-authors, the results were interpreted and the figures were prepared. Dr Winfried Otten wrote the first draft of the manuscript, which was edited by Dr Ellen Kanitz and me before it was submitted to the journal.

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Research paper

It's getting hairy – External contamination may affect the validity of hair cortisol as an indicator of stress in pigs and cattle

Winfried Otten[a,*], Susen Heimbürge[a], Ellen Kanitz[a], Armin Tuchscherer[b]

[a] Leibniz Institute for Farm Animal Biology (FBN), Institute of Behavioural Physiology, Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany
[b] Leibniz Institute for Farm Animal Biology (FBN), Institute of Genetics and Biometry, Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany

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ABSTRACT

Hair cortisol concentration (HCC) is increasingly used for the assessment of enhanced hypothalamic–pituitary-adrenal (HPA) axis activity, e.g., caused by repeated or long-term stressful conditions or pathologies. However, there is still a lack of knowledge regarding the mechanisms and sources of cortisol incorporation into the hair and possible confounding factors, especially in non-human animals. Farm animals are usually kept under confined housing conditions, have close contact with each other and with soiled environments and may thus be exposed to contamination with urine, feces and saliva, which are known to contain substantial concentrations of cortisol or its metabolites. Therefore, the aim of our study was to investigate the impact of contamination with urine, feces and saliva on the cortisol concentration in the hair of pigs and cattle. In an *in vitro* experiment, hair strands of 12 pigs and 12 cattle were repeatedly contaminated with urine and saliva, containing either low or high cortisol concentrations, or with the feces of the respective species and were compared with hair treated with water or untreated hair. Contamination was performed over 20 days for two hours daily. Thereafter, all samples were washed, ground, extracted and analyzed for HCCs following the same protocol. Our results showed that contamination with urine caused a considerable and concentration-dependent increase in HCCs in both species. Saliva had a comparable effect only in cattle. In addition, the treatment with water led to a reduction in the cortisol concentration of porcine hair, whereas contamination with feces caused an increase in HCC only in cattle. Our findings provide evidence that contamination of hair with cortisol-containing body fluids causes incorporation of cortisol into the hair shaft, probably via diffusion depending on the concentration gradient. In that case, cortisol in hair derived from contamination cannot be distinguished from cortisol originating from blood. Thus, contamination may affect the validity of hair cortisol as an indicator of HPA axis activity and cannot be prevented by decontamination protocols prior to analysis.

1. Introduction

The analysis of hair cortisol concentrations (HCCs) for the assessment of hypothalamic–pituitary-adrenal (HPA) axis activity in humans and animals has increasingly gained attention because it enables the reflection of stress over longer periods of time, and sampling procedures are easy and minimally invasive (Greff et al., 2019; Heimbürge et al., 2019). Sources of variation in HCC by individual, contextual and stress-related factors have been extensively studied in humans and animals (Heimbürge et al., 2019; Stalder et al., 2017); however, there is still a lack of knowledge on the mechanisms of cortisol incorporation into the hair and confounding sources in addition to systemic cortisol. In animals, there might be particular confounding factors due to specific environmental and housing conditions, which may facilitate external contamination of the hair. Farm animals are usually kept under confined housing conditions, have close and prolonged contact with soiled environments and with each other and may thus be repeatedly exposed to contamination with urine, feces and saliva known to contain substantial concentrations of cortisol or its metabolites (Mormède et al., 2007; Sheriff et al., 2011; Palme, 2012). A study by our group recently showed that hair samples from caudal body regions and older hair segments exhibited increased HCCs in pigs and cattle (Heimbürge et al., 2020a). This may indicate that the soiling of hair in body regions, which are more exposed to excrements, and the longer duration of exposure in older hair segments may facilitate incorporation of external cortisol from contaminants into the hair. Therefore, the aim of our study was to investigate the impact of contamination with urine, feces and saliva as potential confounding factors on the cortisol

* Corresponding author at: Institute of Behavioural Physiology, Leibniz Institute for Farm Animal Biology (FBN), Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany.
E-mail address: otten@fbn-dummerstorf.de (W. Otten).

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concentration in the hair of pigs and cattle. An *in vitro* experiment was conducted in which hair strands of pigs and cattle were repeatedly contaminated with urine or saliva of the respective species, containing either low or high cortisol concentrations. Additional hair strands were contaminated with feces or treated with water and compared with untreated hair. Thereafter, all samples were washed, ground and extracted according to a standard protocol and analyzed for HCCs.

2. Material and methods

2.1. Animals and hair sampling

The procedures performed in this study involving animal handling and hair sampling were in accordance with the German Animal Protection law and were approved by the relevant authority (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei, Mecklenburg-Vorpommern, Germany). Hair samples were taken from 12 German Landrace sows kept in the Experimental Pig Unit of the Leibniz Institute for Farm Animal Biology (FBN Dummerstorf) and from 12 Holstein Friesian cows in the neighboring Landesgut Dummerstorf.

Hair samples were carefully clipped from the back region in both species by an electric hair clipper (Wahl Professional KM 10, Mosonmagyaróvár, Hungary) as close to the skin as possible (trimmer blade: Oster Cryogen-X Nr. 50 (0.2 mm), Boca Raton, USA) and were collected by hand. Dirty areas were avoided, and from cows, only black hair was used. Hair samples were dried, weighed and stored light protected at room temperature until further processing.

2.2. Sampling and preparation of urine, saliva and feces

2.2.1. Pigs

For the *in vitro* experiment, urine was collected during a separate study of our group in which sows either received an i.m. ACTH administration or were left untreated. Samples were taken from one sow 3–4 h after ACTH administration to obtain urine with high cortisol concentrations (U_H). In addition, urine samples were collected from four untreated control animals for the preparation of a urine pool with low cortisol concentrations (U_L).

Saliva was sampled from gilts using hanging cotton ropes in pens with six animals for one hour. Afterwards, ropes were cut into pieces, centrifuged, and saliva was pooled and analyzed for cortisol concentration. As the salivary cortisol concentration indicated stress levels, probably caused by the excitement of the animals playing with the cotton ropes, an aliquot of the pooled saliva was used as saliva with a high cortisol concentration (S_H). A second aliquot was diluted with distilled water (1:2, v/v) and served as saliva with a low cortisol level (S_L).

Samples of the U_H, U_L, S_H and S_L pools were analyzed for cortisol concentrations, aliquoted and frozen at −20 °C. The analyses of the pooled porcine samples revealed cortisol concentrations in the urine pools of 26.5 ng/ml (U_L) and 324.6 ng/ml (U_H), as well as 4.5 ng/ml (S_L) and 14.3 ng/ml (S_H) in the saliva pools.

Feces were collected from three untreated sows, and a pool of 150 g (50 g from each sow) was prepared and frozen at −20 °C until experimental use.

2.2.2. Cattle

Urine and saliva samples of cattle were taken from animals of a separate experiment in which cattle received either repeated i.m. ACTH administration or saline (Heimbürge et al., 2020b). Sampling was performed 3–4 h after ACTH administration to obtain samples with elevated urinary (U_H) and salivary (S_H) cortisol levels. Accordingly, samples from untreated control animals were used for the collection of samples with low urinary (U_L) and salivary (S_L) cortisol concentrations. Urine was collected with containers during urination, and saliva sampling was achieved with cotton swabs. Samples from ACTH and control animals were pooled separately, analyzed for cortisol concentrations, aliquoted and frozen at −20 °C. In the pooled bovine samples, cortisol concentrations were 27.3 ng/ml (U_L) and 694.8 ng/ml (U_H), as well as 0.44 ng/ml (S_L) and 7.2 ng/ml (S_H). Feces were collected from three untreated cattle, and a pool of 150 g (50 g from each cow) was prepared and frozen at −20 °C until experimental use.

2.3. Contamination protocol

To ensure similar starting conditions for all hair samples, only samples with HCCs of 50–70 pg/mg in pigs and 6–10 pg/mg in cattle were used. Hair samples of pigs and cattle were either repeatedly contaminated with urine (U_H or U_L), saliva (S_H or S_L) or feces of the same species, treated with water or left untreated. Repeated contamination was performed five days per week over a period of four weeks (20 times in total). At the beginning, seven portions of 200 mg hair from each animal were weighed in 5-ml glass tubes. The contamination started by adding 2 ml of liquid (U_H, U_L, S_H, S_L, fecal suspension (200 mg/ml) or distilled water) to the tubes. The fecal suspension was previously prepared by mixing 400 mg of feces with 2 ml water. Then, the tubes were shaken for 2 h, whereby they were turned approximately 90° every 30 min to allow consistent wetting of the whole hair sample. Afterwards, the liquid was discarded, and the hair was pulled out of the tubes and dried on plastic trays light protected and at room temperature until the next day. The contamination procedure was repeated once on every treatment day. After the treatment period, hair samples were dried, weighed and stored in the dark at room temperature until analysis. Due to the limited volume of the saliva pools in both species, contamination with S_H and S_L was performed with hair samples of only six instead of 12 animals.

2.4. Analysis of cortisol in hair, saliva and urine

Extraction and analysis of hair cortisol was performed using the procedures described by Heimbürge et al., 2020. Briefly, hair samples were washed twice with isopropanol, dried at room temperature, flash-frozen in liquid nitrogen and pulverized using a ball mill (MM 400, Retsch GmbH, Haan, Germany). Thereafter, cortisol was extracted with methanol and analyzed in duplicate using an ELISA kit for salivary cortisol detection (Demeditec Diagnostics GmbH, Kiel, Germany). The sensitivity of the assay was 0.8 pg/mg for cattle hair and 1.1 pg/mg for pig hair. The intra- and inter-assay coefficients of variation (CV) were 4.3% and 7.8% for cortisol analyses in the hair of cattle and 4.5% and 6.8% for hair cortisol in pigs, respectively.

After thawing, saliva samples were centrifuged at 2,500 g for 5 min, resulting in a clear supernatant with low viscosity. The analysis of cortisol concentrations in 50 µl saliva samples was performed in duplicate using an ELISA kit for salivary cortisol detection (Demeditec Diagnostics GmbH, Kiel, Germany) as described by Goursot et al. (2019). In pigs, the sensitivity of the assay was 0.08 ng/ml, and the intra- and inter-assay CV were 3.4% and 7.2%, respectively. In cattle, the sensitivity of this assay was 0.06 ng/ml, and the intra- and inter-assay CV were 4.2% and 8.6%, respectively.

The analysis of cortisol concentrations in 10 µl urine samples was performed in duplicate using an ELISA kit for urinary cortisol detection (DRG Instruments GmbH, Marburg, Germany) according to the instructions of the manufacturer. The sensitivity of this assay is indicated with 2.95 ng/ml and the intra- and inter-assay CV with 6.5% and 7.2%, respectively.

2.5. Statistical analyses

The data analysis was performed using SAS software, Version 9.4 for Windows (SAS Institute Inc., Cary, NC, USA). Descriptive statistics and tests for normality were calculated with the UNIVARIATE procedure of Base SAS software. Data of hair cortisol concentrations were

approximately normal distributed in both species and were analyzed by repeated measurement analyses of variance (ANOVA) using the MIXED procedure of SAS/STAT software. The repeated measurement ANOVA model for both species contained the fixed factor treatment (levels: U_H, U_L, S_H, S_L, feces, water, untreated). Different contaminations of hair samples from the same animal were taken into account by the repeated statement of the MIXED procedure using the SUBJECT = animal option to define the blocks of the block-diagonal residual covariance matrix, and the TYPE = CS option to define their compound symmetry covariance structure. Least squares means (LSM) and their standard errors (SE) were estimated and pairwise comparisons were made using the Tukey-Kramer test. Effects and differences were considered as significant if $p < 0.05$. All results are expressed as LSM ± SE.

3. Results

The results from the ANOVAs revealed a significant effect of treatment on cortisol concentrations in the hair of pigs and cattle (both: $p < 0.001$). In pigs, pairwise comparisons indicated that repeated contamination with urine significantly increased HCCs in a concentration-dependent manner compared with those of the untreated control. Contamination with saliva had no significant effect. However, repeated treatment of hair samples with water and the fecal suspension significantly decreased the HCCs in pigs compared to those in the untreated control (Fig. 1). In cattle, contamination with urine, saliva and feces significantly increased HCCs, which was dependent on the cortisol concentration in the urine and saliva. Repeated treatment with water had no significant effect on cortisol concentrations compared to untreated control in bovine hair (Fig. 2).

4. Discussion

To the best of our knowledge, this is the first study to demonstrate the effects of contamination with urine, saliva and feces as potential interfering factors for increased HCCs in farm animals. We showed that repeated contamination of hair with urine increased HCCs in pigs and cattle, and the magnitude of the increase depended on the cortisol concentration in the urine. Contamination with saliva, which contained less cortisol than urine, had no significant effect on cortisol levels in pig hair, whereas in cattle, it also increased HCCs in a concentration-dependent manner. These diverging results for contamination with saliva in hair of pigs and cattle may be caused by differences in hair length

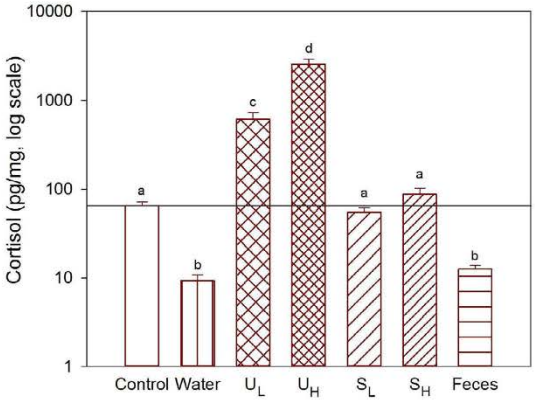

Fig. 1. Effects of repeated treatments of porcine hair samples with water, urine, saliva and a fecal suspension compared with untreated control samples. Treatments with urine (U) and saliva (S) were performed with low (L) and high (H) cortisol concentrations. Data are presented as LSM ± SE, and significant differences are indicated by different letters ($p < 0.05$).

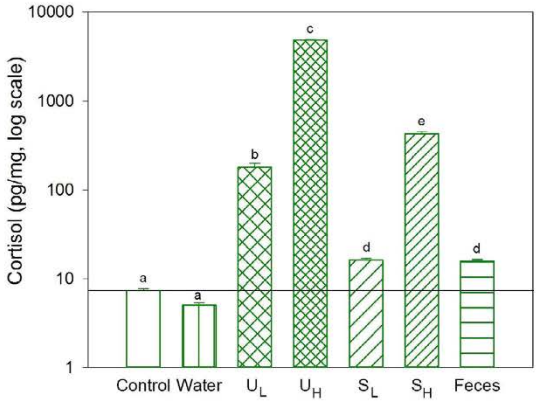

Fig. 2. Effects of repeated treatments of bovine hair samples with water, urine, saliva and a fecal suspension compared with untreated control samples. Treatments with urine (U) and saliva (S) were performed with low (L) and high (H) cortisol concentrations. Data are presented as LSM ± SE, and significant differences are indicated by different letters ($p < 0.05$).

and in weight of single hairs between the two species. Because hair of cattle is shorter and thinner than pig hair, samples of the same weight contain more single hairs in cattle. Thus, a higher total surface and more cut surfaces may facilitate the incorporation of cortisol into the hair shaft in cattle. In addition, the concentration gradient between salivary cortisol (S_H) and hair cortisol was much higher in cattle than in pigs, which may also contribute to the more pronounced increase of HCC in cattle. Hair cortisol concentrations were decreased by contamination with feces in pig hair but increased in the hair of cattle with fecal contamination. This finding may be due to different cortisol excretions via feces in pigs and cattle. Palme et al. (1996) showed that labeled cortisol was excreted via feces to a lesser extent in pigs compared with that in ruminants. To achieve standardized contamination in our experiment, feces were suspended in water. The presumably lower cortisol concentration in the fecal suspension of pigs compared with that in cattle may therefore have caused a dilution effect, similar to the results observed in samples treated with water.

Further indications for contamination effects by urine, saliva or feces on HCCs may also be derived from findings in other animal studies. It was shown that high HCCs in cows were associated with soiling of the lying area in the cowshed with dung and with the presence of dirty flanks of the animals (Sharma et al., 2019). Several reports showed that HCCs differ between body regions within the same animal, indicating that caudal regions, such as the tail and the back region, which may be more exposed to excrements than cranial regions, exhibit elevated HCCs (Burnett et al., 2014; Casal et al., 2017; Heimbürge et al., 2020a; Moya et al., 2013). In a recent study by our group, we investigated the effects of repeated i.m. ACTH administrations on hair and saliva cortisol concentrations in pigs and cattle (Heimbürge et al., 2020b). Animals were kept in mixed groups of both ACTH- and saline-treated animals, and although saliva cortisol levels increased only in ACTH animals, the HCCs increased in pigs of both treatments after the administration period. This finding indicated that in pigs, a possible cross-contamination from the ACTH-treated to the control animals occurred, most likely caused by their contact with the urine and saliva of the ACTH-treated animals, e.g., by lying in dung or by social interactions. This assumption is supported by a study of Macbeth et al. (2010), who reported elevated HCCs in brown bears captured in culvert traps compared with those in brown bears captured via other methods. The authors assume that soiling of hair by urine, feces and bait in the trap could facilitate the permeability of hair and the incorporation of cortisol from the contaminants into the hair shaft. In a further, more

controlled experiment of the same group, it was demonstrated that contamination with cortisol-containing urine and feces can increase HCCs of brown bears within 2 h of exposure and that this increase is related to the concentration of cortisol in the slurry (Macbeth, 2013). Thus, it is strongly recommended to take hair samples from animals as clean as possible and to avoid sampling from body sites that may be more exposed to fecal contamination (Ghassemi Nejad et al., 2019a,b; Macbeth, 2013). The choice of the appropriate body region then depends on the animal species, the specific husbandry environment and the accessibility for sampling. In cattle and pigs kept indoors, this could be the neck, shoulder and back regions, which are outside the reach of the tail and are not affected by soiling when lying down.

In addition to excrements and saliva, sweat could also be a potential interfering factor influencing HCC because it also contains quantifiable amounts of cortisol (Russell et al., 2014). Hence, immersion of human hair in a cortisol solution *in vitro*, mimicking cortisol concentrations of sweat, increased HCCs, and this elevation depended on the immersion time and cortisol concentration in the solution (Grass, 2017; Russell et al., 2014). Contamination by cortisol crème was also found to markedly increase HCCs up to 30 days after application, even with regular hair washing several times a week (Wang et al., 2019). It is important to note that in the aforementioned studies and in our experiment, established hair washing protocols did not eliminate contamination effects, indicating that external cortisol had been incorporated into the hair matrix and could not be removed by washing (Macbeth et al., 2010; Russell et al., 2014; Wang et al., 2019).

In general, it is assumed that the main mechanism of incorporation of organic molecules into the hair is passive diffusion from blood vessels (Pragst and Balikova, 2006). Additionally, substances can be deposited from the external environment as proposed by the multi-compartment model (Henderson, 1993). Lipophilic molecules, such as cortisol and other steroids, can easily penetrate membranes and diffuse according to the concentration gradient in matrix cells (Pragst and Balikova, 2006). Water causes swelling of hair (Kidwell and Smith, 2007; Robbins, 2002) and thus may facilitate permeability and diffusion of cortisol from aqueous solutions, such as urine, saliva and sweat, into the hair matrix. Our results and the data from other contamination studies show evidence that cortisol deposition from urine, saliva and sweat occurs via diffusion depending on the concentration gradient between the contaminating solution and the hair. When the hair dries, contaminants might be tightly incorporated and cannot be removed by detergents, such as isopropanol, which does not cause hair swelling (Eser et al., 1997).

Interestingly, incorporation of contaminants may be facilitated by damage to the hair shaft (Kidwell and Smith, 2007). In this regard, hair with cut surfaces from shaving, as was the case in our study, revealed higher HCCs after exposure to artificial cortisol-containing solutions than those of hair without cut surfaces (Grass, 2017). This finding indicates that a higher degree of structural hair damage can result in an enhanced incorporation of external cortisol into the hair shaft. An additional *in vivo* experiment by Grass (2017) indicated that cortisol incorporation from an artificial sweat solution is more pronounced in distal than in proximal hair segments. Assuming more structural hair damage in distal and therefore older hair segments, e.g., by hair washing, sunlight exposure or natural friction of hair cuticle cells (Dawber, 1996; Richena and Rezende, 2016), this may lead to a higher susceptibility to external contamination by cortisol-containing liquids. This assumption is supported by results from a previous study by our group in pigs and cattle (Heimbürge et al., 2020a) and other studies in sheep, black bears and chimpanzees (Fürtbauer et al., 2019; Malcolm et al., 2013; Yamanashi et al., 2016), where increased cortisol concentrations from proximal to distal hair segments were found.

Our results also indicate that, at least in pig hair, cortisol can be removed from the hair shaft by frequent washing with water. This finding is in line with results from *in vitro* experiments showing that hair treatments with water alone or with a shampoo solution caused HCC loss (Hamel et al., 2011; Li et al., 2012). However, a washing step with water prior to the washing of hair samples with alcohol is preferred in some studies to reduce hydrophilic contaminants from the hair surface (Burnett et al., 2014; Bacci et al., 2014; Cooke et al., 2017). In our study, HCC in untreated control samples was higher in pigs (65 pg/mg) than in cattle (7 pg/mg) and decreased to comparable levels after washing with water (9 vs. 5 pg/mg in pigs and cattle, respectively). Thus, the higher wash-out of cortisol in pig hair may be caused by the higher concentration gradient during washing. Enhanced hair damage may facilitate an increased removal of cortisol from the hair shaft by diffusion processes during hair washing, frequent contact with water or washing procedures during analysis (Manenschijn et al., 2011). In line with this assumption, observations from our experiments and other research revealed that pig hair often show split ends (Feder, 1980; Mohan et al., 2015), which may contribute to the different wash-out effect in pigs and cattle. Numerous studies describe a decline in cortisol concentrations from proximal to distal hair segments in different species (e.g., humans: Kirschbaum et al., 2009; chimpanzees: Carlitz et al., 2015; cattle: Nedić et al., 2017). Thus, it can be suggested that increased hair damage, especially in the older, distal segments, may facilitate both incorporation and removal of cortisol *in vivo* depending on the frequency and duration of contamination with cortisol-containing body fluids and water.

5. Conclusions

The results of our *in vitro* study revealed that repeated contamination of hair with urine causes considerable and concentration-dependent increases in HCCs in pigs and cattle. Contamination with saliva had a similar effect only in cattle but to a lesser extent. A wash-out of cortisol was found in porcine hair when treated with water or feces diluted with water. Our findings provide the first evidence that contamination of porcine and bovine hair with cortisol-containing body fluids, such as urine and saliva, may cause incorporation of external cortisol into the hair shaft, probably via diffusion depending on concentration gradients. Swelling of hair by aqueous contaminants may facilitate these diffusion processes. Finally, incorporated cortisol originating from contamination cannot be distinguished from cortisol originating from blood; thus, contamination may impact the validity of hair cortisol as an indicator of HPA axis activity. Consequently, it is strongly recommended to take hair samples as clean as possible and to avoid sampling from body sites that may be more exposed to fecal contamination. Further studies are necessary to investigate whether structural damage of hair, e.g., that induced by aging or by cut or broken surfaces, may enhance incorporation or wash-out of cortisol.

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CRediT authorship contribution statement

Winfried Otten: Conceptualization, Methodology, Investigation, Writing - original draft, Funding acquisition. **Susen Heimbürge:** Conceptualization, Methodology, Investigation, Writing - review & editing. **Ellen Kanitz:** Conceptualization, Methodology, Writing - review & editing, Funding acquisition. **Armin Tuchscherer:** Formal analysis.

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5 General discussion

As detailed discussions of the results have already been conducted separately in each publication, this general discussion will comprehensively discuss the most important findings of our studies. Based on the aims of my thesis and the specific approaches of the studies, the general discussion has two main sections: the dependence of hair cortisol levels on influencing factors and the impact of long-term stress. Therefore, I will first highlight the basic determinants that may cause fluctuations in systemic cortisol concentrations independent of stress and that may affect hair cortisol concentrations. In addition, the impact of hair-specific factors and of external contamination and elimination by washout on hair cortisol levels will be discussed. The second part of the general discussion comprises the applicability of hair cortisol as an indicator of long-term stress in cattle and pigs. Background information on the application of the ACTH model for the simulation of long-term stress is given, and models for the time course of cortisol incorporation into the hair of cattle and pigs are presented. The influence of hair sample type and sampling time on the incorporation of cortisol will also be evaluated. At the end of each main section, implications are presented and recommendations for the use of hair cortisol are given. Finally, possible future research approaches and applications of hair cortisol measurements under practical and experimental conditions are derived.

5.1 Influencing factors on hair cortisol concentrations in cattle and pigs

5.1.1 Impact of animal-based, seasonal and hair-specific factors

The literature review identified various factors that may influence cortisol concentrations in animal hair. Therefore, our experiments investigated the animal-based determinants in cattle and pigs, such as age and sex, hair-specific characteristics, such as hair colour, body region, hair segment age and season.

We found elevated cortisol levels in the hair of newborn cattle and pigs as well as adult sows. Newborn calves showed hair cortisol levels approximately nine times higher and piglets three times higher than the infantile, juvenile and adult age groups of their species. Since the hair of newborn animals is predominantly grown in utero (MEYER and GÖRGEN 1986), this increase may be due to elevated maternal and foetal cortisol levels during late gestation and around birth (KANITZ et al. 2012, OTTEN et al. 2013). Newborn animals also exhibit higher total cortisol and lower corticosteroid-binding globulin levels in plasma, which may lead to higher concentrations of unbound cortisol (KANITZ et al. 2012, GRANT et al. 2017) and thus to a higher incorporation of cortisol in the hair. A few weeks after birth, shedding of birth coat occurs (SLEE 1963, LING 1970), which is probably the reason for the marked decrease in HCC at this time. The results also revealed a higher HCC in sows than in younger age groups. This effect may be due to lower sensitivity of the

HPA axis with age, which has been found in older age groups of other species (SAPOLSKY et al. 1986, DETTENBORN et al. 2012, FOURIE et al. 2015). However, this age-related increase was observed in pigs but not cattle, possibly because hair samples were collected from sows during the insemination period. Previous studies have shown that sows exhibit higher HCCs during the insemination period than at other stages of the reproductive cycle, probably also due to the preceding increase of cortisol levels during late pregnancy and around birth (BACCI et al. 2014, personal data).

Studies of various species have found hair cortisol levels to depend on sex (humans: STALDER et al. 2017, pigs: BERGAMIN et al. 2019, horses: MEDILL et al. 2015, goats: DULUDE-DE BROIN et al. 2019, muskoxen: DI FRANCESCO et al. 2017). These differences may be caused by varying systemic cortisol concentrations due to sex-specific hormonal changes in the stress response (RAVEN and TAYLOR 1996, TURNER et al. 2012). Furthermore, males and females may exhibit divergent behavioural patterns, such as mating rituals and territorial fights, which could also lead to different cortisol release (BERGMAN et al. 2005). In our study, the sex of the cattle and pigs had no influence on their HCCs. However, hair samples from animals of both sexes could only be collected from calves and piglets shortly after birth because in later age groups only castrates were available. Thus, animals in our study were not fully influenced by sexual hormones, as is the case with intact, adult animals. A reason for the missing differences between females and males in our study could therefore be the lack of sample material from sexually mature animals.

Regarding a potential effect of the season of the year on hair cortisol concentrations, we observed alterations only in cattle hair, which showed higher cortisol levels in winter than summer. The seasonality of HCC can be caused by numerous influencing factors that vary over the year, such as temperature, daylight and seasonal sexual, territorial and nutritional behaviours (BOSWELL et al. 1994, VENTRELLA et al. 2018). In our study, the length of daylight was chosen as the decisive factor for the classification of summer and winter, so hair samples were taken around the summer and winter solstice. Since the pigs were kept indoors all year round, changes in daylight length and ambient temperature were less likely to affect the incorporation of cortisol into hair. However, the cattle in our study were kept under freely ventilated loose housing conditions and thus may have been more affected by changing environmental conditions. Previous studies have shown that both natural sunlight and ultraviolet (UV) radiation can degrade hair cortisol (GRASS et al. 2016, WESTER et al. 2016). Thus, the lower hair cortisol levels in cattle hair during summer may be a result of this photodegradation. However, since there was no effect of seasonality in pigs and even in cattle, the quantitative difference in HCC was comparatively small (winter: 8.6 pg/mg, summer: 6.2 pg/mg), the impact of seasonality under such housing conditions seems to be low.

In addition to animal-based and seasonal factors, the impact of various hair-specific factors on HCCs, such as hair growth rate, body region, hair colour and hair segment, was investigated. For better interpretation of HCCs in different body regions, the growth rates of hair from the tail,

shoulder, back and neck regions were determined. Hair growth rates in the tail tip were approximately 5.6 times higher in cattle and 2.0 times higher in pigs than in other regions. These differences may be caused by variations in blood supply or diverging hair types in the examined regions (HARKEY 1993, MADEA 2004). In addition, we also found differences in hair cortisol levels between body regions. Cortisol concentrations in hairs in the tail tip were three times higher than concentrations in hair on the shoulder and back in cattle and up to six times higher than in the neck and back regions in pigs. Possible reasons for these differences in cortisol incorporation include the predominant hair colour and hair type in the body region as well as external factors: back regions could be more exposed to weather conditions such as sunlight or rain, degrading or washing out the cortisol in the hair (STALDER and KIRSCHBAUM 2012, GRASS et al. 2016, WESTER et al. 2016). However, regions such as the tail or abdomen could be more exposed to soiling by urine and faeces, which can confound hair cortisol levels due to contamination (MACBETH et al. 2010).

The results concerning the influence of hair colour on hair cortisol levels in cattle and pigs revealed higher cortisol concentrations in black samples than white samples in both species. However, the quantitative difference in cortisol between black and white hair was smaller than the impact of other influencing factors, such as age and body region. To date, the underlying mechanisms causing the cortisol differences between hair colours have not been fully deciphered. Presumably, the amount of melanin in the hair significantly affects both the incorporation and degradation of cortisol in the hair shaft. Black hair could lead to higher skin temperatures and thus increased blood flow in the skin, promoting the incorporation of cortisol via blood vessels (BURNETT et al. 2014). Furthermore, melanin itself appears to facilitate the incorporation of lipophilic substances, such as steroids (PRAGST and BALIKOVA 2006). However, our results showed an influence of hair colour in natural but not newly regrown hair, suggesting that cortisol differences between hair colours may be due to external influences rather than a variation in cortisol incorporation. Natural hair is older and therefore exposed to external influences for longer periods than newly grown hair. Hence, this effect may only be observed in samples containing older hairs. Consistent with our results, an effect of hair colour was found when studying the photodegradation of drugs in hair with higher degradation in bright hair than in dark hair (FAVRETTO et al. 2014). Eumelanin pigments in black hair can shield and absorb UV radiation, thereby protecting proteins and other substances such as drugs and GCs from photodegradation (HOTING et al. 1995, FAVRETTO et al. 2014). Thus, the higher degradation of cortisol in white hair may contribute to lower HCC in samples of this hair colour.

In addition, the influence of the age of hair segments on hair cortisol concentrations was examined. For this purpose, the tail hair of cattle was cut into three 2-cm segments and hair from the back regions of pigs was cut into four 1-cm segments. The results showed a marked increase in HCC from the proximal to the distal hair segment. Cortisol levels in the most distal segments were

approximately 11 times higher in pigs and 2.5 times higher in cattle than in the proximal segments. External influences, such as sunlight, hair washing and natural friction, can damage the cuticle cells of the hair, resulting in progressive surface destruction of the hair shaft with increasing hair length (DAWBER 1996, RICHENA and REZENDE 2016). This may facilitate the incorporation of external cortisol into the hair shaft and the elimination of incorporated cortisol from the hair, such as by hair washing (GRASS 2017). Consistent with this assumption, human hair often shows a decrease in HCCs from proximal to distal segments, which is probably caused by frequent hair washing and other hair treatments (STALDER et al. 2017). Because systemic cortisol can be excluded as a source of the observed increase of HCCs along the hair shaft in our studies, external influences due to species-specific husbandry conditions and behaviour are likely. The main reason for increased HCC along the hair shaft is presumably the enhanced, prolonged contamination with cortisol-containing fluids, such as urine and saliva.

5.1.2 Impact of contamination and elimination by washout

The findings that caudal body regions and distal hair segments exhibit elevated HCCs led to the hypothesis that cortisol contained in body fluids, such as urine, saliva and faeces, may be incorporated into the hair shaft by external contamination. The result that control pigs showed a similar increase of HCC as ACTH animals after the treatment period also supports this hypothesis. Since ACTH and control animals were kept in mixed groups under conventional housing conditions on slatted floors, hairs from control animals could be contaminated with body fluids from ACTH-treated animals by lying on soiled floors or by social interactions. Thus, HCC in control pigs might be elevated by the diffusion of external cortisol derived from the urine, saliva or faeces of ACTH pigs. To test this hypothesis, an *in vitro* experiment was performed in which bovine and porcine hair samples were repeatedly contaminated over four weeks with either urine or saliva containing two different concentrations of cortisol, contaminated with faeces or washed with water and then compared with HCCs in untreated control samples. The contamination of hair with urine resulted in increased cortisol levels in the hairs of both cattle and pigs, whereas saliva, which contained less cortisol than the urine, increased HCC only in bovine hair. Furthermore, the results show that the incorporation of cortisol into hair depends on the cortisol concentration in urine and saliva. Therefore, these findings confirm our hypothesis and provide preliminary evidence that contaminating of hair with body fluids increases HCCs by a concentration-dependent incorporation of external cortisol from the fluids into the hair.

Further, the results show that contamination with a faecal solution leads to an increase in cortisol concentrations in bovine hair but to a decrease in porcine hair. This may be due to species-specific differences in cortisol excretion in faeces, as a previous study showed that pigs excreted less cortisol in their faeces than ruminants (PALME et al. 1996). Furthermore, the faecal samples

were diluted with water, which in addition to the already low cortisol concentration in pig faeces, can even lead to washout. Repeated treatment with water alone eliminated cortisol from the hair shaft only in pigs. In contrast, in cattle, cortisol levels in water-treated samples remained unchanged compared to untreated hair samples. However, the cortisol concentration in untreated porcine hair was markedly higher than in bovine hair, resulting in a more pronounced concentration gradient between water and hair in the porcine samples, which may explain these different findings. Consistent with the observed washout effect in porcine hair, previous *in vitro* studies have shown that hair treatment with water or shampoo solution can decrease HCC (HAMEL et al. 2011, LI et al. 2012). Contact with aqueous liquids causes swelling of the hair (ROBBINS 2002, KIDWELL and SMITH 2007) and can thus facilitate the incorporation and elimination of dissolved substances into and from the hair shaft. To avoid possible surface contamination by external cortisol, hair samples are usually washed before analysis. In general, as in our study, alcohol is used to remove lipophilic substances from the hair surface, as they hardly cause swelling.

Discrepancies in the contamination effects between bovine and porcine hair could also be due to species-specific differences in hair structure. Bovine hairs are shorter, thinner and lighter than porcine hair, so bovine hair samples of the same weight contain more single hairs, and more single hairs result in a larger total surface area of the entire sample, which could increase contamination effects. It has been shown that hair with cut surfaces from shaving reveal higher HCCs after exposure to artificial cortisol-containing solutions than intact hair (GRASS 2017). Cut hair ends are likely potential entry points for cortisol due to their damaged structure. Therefore, it is possible that in our study, cortisol incorporation in bovine hair samples was facilitated by more cut hair ends than in the porcine hair samples. The concentration gradient between salivary cortisol and hair cortisol was also markedly higher in cattle than in pigs, which may contribute to the more pronounced increase in HCC in cattle.

Overall, the *in vitro* experiment indicates that the contamination of hair with urine and saliva can strongly confound HCCs in pigs and cattle. The impact of external contamination and elimination by washout in farm animals seems to depend on the cortisol concentration of the contaminant as well as on species-specific housing conditions and behavioural patterns, such as lying on soiled floors and social interactions. In pigs and cattle, urine contributed most to rising hair cortisol, followed by saliva, whereas the influence of faecal contamination on cortisol incorporation seemed only minor. Finally, a clear elimination of cortisol by water treatment was observed in pigs.

5.1.3 Implications

Our studies of cattle and pigs were the first to investigate various influencing factors on hair cortisol concentrations in farm animals to this comprehensive and comparative extent. The results showed variations in HCCs depending on the age of the animal, the body region of sampling, the hair segment and the hair colour. Age, body region and hair segment had great impacts, whereas the influence of hair colour was small. However, it is advisable to standardise as many influencing factors as feasible when comparing HCCs in different animal groups or within the same animal. Furthermore, evaluations of HPA axis activity or stress load, such as for welfare assessment, must consider that there are life periods in which systemic cortisol concentrations, and thus hair cortisol levels, are physiologically elevated, such as in newborn animals.

The present findings reveal a pronounced effect of external contamination and a potential influence of water treatment on hair cortisol levels. To avoid these effects, it is advisable to shave hair from obviously dry, clean body regions. Choosing the appropriate body region depends on the animal species, the specific husbandry environment and the accessibility for sampling. For cattle and pigs kept indoors, the sampling regions could be the neck, shoulder or back, which are out of reach of the tail and are not affected by soiling when the animals lie down. It is advantageous to use only the most proximal segments of the hair samples to avoid the confounding effect of contamination or washout. Similarly, the shave-reshave procedure and thus the analysis of only newly regrown hairs could be applied.

To further assess the impact of external contamination on the incorporation of cortisol into the hair shaft in farm animals, it would be useful to perform an *in vivo* experiment similar to the *in vitro* study. It is possible that due to the close connection of the hairs in the coat and the lack of cut ends, less of the surface contacts cortisol-containing contaminants and therefore less cortisol can penetrate the hair shaft. In addition, the hairs are constantly kept supple by sebum, which makes aqueous solutions less adherent and prevents damage to the hair structure (ROBBINS 2002). It would be interesting to find evidence for the assumption that the incorporation of external cortisol is higher in more severely damaged distal hair segments, such as by subsequent segmentation of hair samples collected during an *in vivo* contamination experiment.

5.2 Hair cortisol concentration as an indicator of long-term stress in cattle and pigs

5.2.1 Model for the increased release of systemic cortisol

The adrenocorticotrophic hormone plays a key role in the formation and release of cortisol from the adrenal cortex, thereby influencing systemic cortisol levels in the blood (SPENCER and DEAK 2017). For this reason, administrations of ACTH are commonly used to simulate the stress-related activity of the HPA axis (KERSEY and DEHNHARD 2014). Since it is assumed that the main pathway of cortisol incorporation into the hair shaft is diffusion from the bloodstream (STALDER and KIRSCHBAUM 2012), ACTH applications are useful for investigating the relationship between increased systemic cortisol concentrations and hair cortisol levels. As shown in previous studies, repeated administrations of ACTH increase the activation of the HPA axis and the subsequently elevated incorporation of cortisol into the hair shaft (GONZÁLEZ-DE-LA-VARA et al. 2011, DULUDE-DE BROIN et al. 2019). Therefore, repeated ACTH applications are a suitable model for the validation of HCC as a retrospective indicator of long-term stress. In the present study, the animals received either 2 ml of ACTH solution (100 IU Synacthen Depot) or 2 ml of saline intramuscularly every second day over four weeks. This experimental protocol was selected based on dose-response curves and results described in previous studies using the same dose in cattle (DOBSON et al. 2000, BIRAN et al. 2015) and pigs (OTTEN et al. 2004, BACKUS et al. 2013). In order to validate the ACTH-application model, we also collected saliva samples, as salivary cortisol levels correlate strongly with systemic blood cortisol concentrations (COOK et al. 1996, NEGRÃO et al. 2004, HELLHAMMER et al. 2009). As a result, significantly increased cortisol levels were observed in saliva samples after ACTH treatment during the application period without alterations in the controls of either species. Therefore, it was concluded that the ACTH-application model used to simulate the cortisol response to chronic intermittent stressors was effective. However, species-specific differences were observed in the magnitude and duration of the salivary cortisol response to ACTH, with a less pronounced reaction in pigs than cattle. Pigs showed elevated cortisol levels up to 6 h after ACTH application and a blunted magnitude, whereas in cattle, salivary cortisol increased for up to 9 h with a higher maximum, so a different effect on the hair cortisol concentration should be expected.

5.2.2 Models for the time course of cortisol incorporation into the hair shaft

To the authors' knowledge, the ACTH study was the first to investigate and compare the effects of long-term stress on cortisol levels in different hair sample types at various sampling times in cattle and pigs. As part of this ACTH study and based on average hair growth rates and hair follicle depth in cattle and pigs, hair growth models were developed to determine the sampling times at which the highest HCCs are expected (see Fig 5. and Fig. 6 of Study 3).

Our results revealed hair growth rates of approximately 3.5 mm per month in the back region in cattle and 7 mm per month in pigs. Additionally, the hair follicle is located about 2 mm below the skin surface in cattle (UDO 1978) and 3.5 mm in pigs (personal data). Based on these data, we hypothesised that the natural hair of ACTH-treated animals would exhibit increased cortisol levels as early as week 4, immediately after the end of treatment. In addition, maximum HCCs were expected four weeks after the end of the treatment period (week 8), when the natural hair samples contained the total amount of cortisol incorporated during the treatment period. We also predicted decreased cortisol concentrations eight weeks after the end of the treatment (week 12) caused by a dilution effect due to newly growing hair with basal cortisol levels. Moreover, natural hair samples contain not only actively growing hairs but also hairs in the catagen and telogen growth stages that cannot incorporate cortisol from the bloodstream. Therefore, these hairs can also dilute HCCs in natural hair samples.

In addition, we collected natural hair from the back region in pigs and the tail tip in cattle eight weeks after the end of the treatment (week 12) to cut them into segments. The lengths of the hair segments were chosen to approximately reflect the hair growth during the entire 12-week experimental period in three segments. Considering the different hair growth rates in the back and the tail tip, the proximal natural hair was cut into 1.5-cm segments in cattle and 1-cm segments in pigs. Taking into account the follicle depth, the hair growth rate and the number of segments, we expected elevated HCC in the most distal hair segments of ACTH-treated animals.

Regrown hair samples were obtained by applying the shave-reshave procedure and therefore mostly contained actively growing hairs. To establish a hair growth model for this hair sample type, we also assumed the hair growth rates and follicle depth as described above. Thus, increased and similar cortisol levels were expected in samples from ACTH-treated animals directly at the end and four weeks after the end of the treatment period (weeks 4 and 8). By week 12, all hair portions with elevated HCC should have been shaved, and no differences between treatment groups were expected.

5.2.3 Impact of hair sample type and sampling time

In cattle, increased cortisol concentrations were observed in all hair sample types (natural, regrown and segments) after ACTH application over four weeks. The cortisol concentrations were highest in regrown hair, which confirmed our hypothesis. In the natural hair of ACTH-treated animals, increased cortisol levels were observed at all sampling times after the treatment period, with the highest cortisol levels four weeks after the end of the treatment (week 8), followed by a decrease of HCC in week 12. In regrown hair samples, cortisol levels were higher in ACTH-treated cattle in weeks 4 and 8 than in the control animals, but the treatment groups had no differences in week 12. In conclusion, these results in cattle fully confirmed our assumptions derived from the hair growth models described above.

The results for salivary cortisol in samples collected during the 4-week treatment period revealed correlations with cortisol levels in both natural and regrown hair. The highest correlation was found between salivary cortisol and cortisol concentrations in regrown hair samples collected in week 4, indicating a strong relation between systemic cortisol levels and the cortisol concentration in this type of hair sample. The regrown hair samples mainly contained hairs that had actively grown within the preceding four weeks and may therefore reflect the systemic cortisol concentration during the previous weeks better than natural hair. Consistent with our results, other studies in goats and cattle have shown the greatest increases in hair cortisol levels after ACTH applications in samples collected within four weeks after treatment but not in later samples (GONZÁLEZ-DE-LA-VARA 2011, ENDO et al. 2018).

The results of the investigations of different hair segments in cattle also confirmed our hypothesis from the hair growth model. Increased hair cortisol levels from proximal to distal hair segments were observed in ACTH-treated animals, not in control animals. Therefore, for the first time in farm animals, hair segmentation appears to be applicable for the assessment of prior stress by creating a retrospective calendar. In conclusion, in cattle, the ACTH application model resulted in the expected increase in cortisol levels in all hair sample types, so hair cortisol could be a suitable indicator of long-term stress in this species.

In pigs, ACTH applications also resulted in increased hair cortisol levels, but only in natural hair in week 4 (the end of treatment) compared with basal levels in week 0. There were no differences in weeks 8 and 12 compared with basal levels. In regrown hair samples, we observed higher HCCs at week 4 (the end of treatment) than week 8. Thus, in contrast to cattle, the hypothesis of increased HCCs due to elevated systemic cortisol levels could only be confirmed in pigs for the sampling time immediately after the end of the treatment. However, like the increase of HCC in ACTH-treated pigs, control pigs also showed elevated hair cortisol levels, and there were no differences between the treatment groups for all hair types at all sampling times. Possible explanations for the lack of differences between the treatment groups could be the less

pronounced cortisol response in pigs after ACTH applications, as mentioned above, and the specific annual hair growth pattern. Cortisol is incorporated by diffusion from blood vessels during the anagen growth phase (STALDER and KIRSCHBAUM 2012). Pigs show a pronounced seasonality of hair follicle activity and undergo one hair growth cycle per year, during which the anagen phase occurs mainly in fall and winter (MOWAFY and CASSENS 1976a, WATSON and MOORE 1990). Therefore, the natural porcine hair samples shaved during the spring and summer seasons in our study may contain many hairs in the catagen and telogen phases that could not incorporate systemic cortisol. Another and more likely explanation for the increase in both treatments is the potential contamination caused by incorporating external cortisol from urine, saliva or faeces, which is explained in detail in Chapter 5.1.2. Due to the conventional housing conditions and species-specific behavioural patterns, the external contamination of hair by soiling appears to be more pronounced in pigs than in cattle. Investigating hair segments in pigs revealed an increase in HCC from proximal to distal segments, like ACTH-treated cattle, but no differences were found between treatments, which was consistent with the results in natural and regrown hair. Thus, increased HCC in the distal segments of control animals could also be due to external contamination. In summary, the results in pigs do not confirm the hair growth models, and the effect of long-term stress could not be assessed by analysing natural, regrown or segmented hairs.

The present results regarding natural, regrown und segmented hair reveal different advantages and disadvantages for each hair sample type. Using natural hair samples requires no preparatory work, and the sampling can be done spontaneously since only one shave is necessary. However, it must be considered that natural hair samples contain hairs that are in all three growth phases, so large portions of the sampled hairs may not be in the active growth phase and thus cannot incorporate systemic cortisol. In addition to this dilution effect, natural hair samples are exposed to external influences for longer periods than regrown samples. The older a hair is, the more its structure can be damaged (DAWBER 1996) and the more easily HCC can be influenced by both contamination and the elimination of cortisol. When using the shave-reshave method, regrown hair samples mainly contain hairs that have actively grown during the previous weeks, so dilution caused by different hair growth phases and external influences on HCC, such as contamination and UV radiation, might be lower. Hence, regrown hair samples can reflect systemic cortisol concentrations better than natural hair samples. Collecting hair segments has the advantage that the sampling time is more flexible than with regrown hair when the hair growth rate and follicle depth are known. However, the sampling time and size of the segments must be adjusted to allow the intended retrospective conclusion. Nevertheless, HCCs in older hair segments can also be influenced by external contamination and elimination, so segmentation should be limited to the most proximal centimetres.

5.2.4 Implications

Based on the comparison of the results in different hair sample types, the use of the shave-reshave procedure can be recommended. For this purpose, an area is pre-shaved (e.g. the back region) before the period of interest and shaved again at the end of that period to collect and analyse regrown hairs. This sample of mainly active growing hairs should be less contaminated and have a lower dilution effect and therefore have a higher retrospective validity than samples of natural and segmented hair. Additionally, at least in cattle and pigs, hair samples should be taken no later than four weeks after the end of a stress period to obtain reliable information of average systemic cortisol concentrations during that period.

The results of the ACTH study on the applicability of hair segments in farm animals show that it might be possible, at least in cattle, to establish a retrospective calendar for the assessment of previous stress periods. With the knowledge of species-specific hair follicle depth and hair growth rate, different segments can be assigned to different preceding time periods. The length of segments and the time of sampling will then also depend on the duration of the period of interest. However, since increased HCC from proximal to distal hair segments was also observed in non-stressed animals, at least in pigs, it may be advisable to use only the most proximal segments.

The time delay between cortisol incorporation and the sampling time is also important to consider. Due to the species-specific hair growth rates and follicle depth in cattle and pigs, an average of 14 days elapses between the incorporation of cortisol at the hair papillae and the time when that part of the hair appears on the skin surface. Considering the hair growth models and based on the present results, the highest HCC may be found in hair samples collected two weeks after the period of interest. However, a more precise clarification of when elevated cortisol levels occur in the hair shaft and above the skin would require an experiment with radio-labelled cortisol, like that conducted by KECKEIS et al. (2012), and more frequent sampling than in our ACTH study. To avoid losing information, we recommend shaving the hair as close to the skin as possible, which is best achieved by shaving against the direction of growth. In addition, plucking the hair should be avoided, as the hair root contains cortisol-producing cells (ITO et al. 2005), and even if these concentrations are low, they could affect HCC results.

5.3 Future perspectives

The results of our studies provide a basis for further investigating the practical applicability of hair cortisol as an indicator of long-term stress. To evaluate the applicability of HCC under practical conditions in cattle and pigs, further research should investigate the impact of stress loads relevant to practice (e.g. high stocking density, high animal/feeding-place ratio and high milk yield). It is of great interest whether the magnitude of these stress loads can also induce a detectable increase in hair cortisol levels. However, this also requires further investigations to determine the physiologically normal range of HCCs in order to differentiate between normal physiological variations and pathological values. Unusually high HCCs would thus have a warning function to indicate particularly high stress levels or pathological conditions. Besides the retrospective assessment of the stress load of an individual animal, pooled samples of many animals could also provide information about housing conditions, possibly indicating stressful influences within a husbandry system or a farm. Additionally, slaughterhouses or dairies could use the information from the hair cortisol monitoring to assess the long-term stress level of a producer's animals, which could be of interest to the processing industry and to consumers, as stress levels can affect meat and milk quality. The use of hair cortisol for the evaluation of stress loads could also become part of welfare assessment and monitoring programmes. It has the advantage that long-term retrospective and individual-based information can be collected in a minimally invasive way, which could improve the comprehensive assessment of housing conditions. A further application for hair cortisol analysis could be its use for stress monitoring in animal experiments. Here, it would be possible to assess the stress load caused by animal treatments more precisely. As the housing conditions in experimental settings are more standardised and more controlled than conventional husbandry conditions, it might also be easier to eliminate confounding factors.

5.4 Conclusions

In general, hair has great potential to serve as an innovative biological sample material. Since repeated activation of the HPA axis increases cortisol incorporated into the hair shaft, the prerequisite for using hair cortisol as an indicator of long-term stress in cattle and pigs is given. Nevertheless, age, body region, hair colour and hair segments are influencing factors that must be considered and standardised whenever possible. Attention must also be paid to the effect of possible external contamination. The present results show that the contamination of porcine and bovine hair with cortisol-containing body fluids, such as urine and saliva, may cause the incorporation of external cortisol into the hair shaft, which may affect the validity of hair cortisol as an indicator of HPA axis activity. Although a potential effect of contamination has been demonstrated *in vitro* in both species, the ACTH study has shown that this is more likely a confounding factor *in vivo* in pigs than in cattle. Therefore, in cattle, hair cortisol concentrations in natural, regrown and segmental hair samples reliably reflected the preceding period with increased systemic cortisol levels, entailing that all three hair sample types can be used to assess long-term stress. Since the highest hair cortisol levels were observed within four weeks after the end of stress, this period is the most appropriate time for hair sampling. Our investigations form a basis for further research on hair cortisol measurements in cattle and pigs. Analysing HCCs offers a minimally invasive, retrospective and animal-based method to measure long-term stress and may improve the comprehensive assessment of animal welfare. Thus, after further validation, hair cortisol analysis could be implemented in animal welfare–monitoring programmes

6 Summary

Author: Susen Heimbürge

Title: Hair cortisol concentration in cattle and pigs: Investigation of influencing factors and the potential as an indicator of long-term stress

Institutions: Institute of Animal Hygiene and Veterinary Public Health, Faculty of Veterinary Medicine, University of Leipzig

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Keywords: hair cortisol, long-term stress, cattle, pigs, ACTH, contamination, animal welfare

Introduction: Farm animals can be exposed to various stressors due to their husbandry conditions, which can impair their health and welfare. Thus, there is interest in the use of minimally invasive methods and animal-based stress indicators as part of welfare assessment. Cortisol in hair is a promising retrospective stress indicator, as a sample reflects systemic cortisol levels of the past weeks or months. Previous studies have shown that long-term stress with elevated cortisol release can be related to increased cortisol incorporation into the hair shaft. However, potential influencing factors that may affect hair cortisol concentrations (HCCs) must be determined before HCC can be applied as a reliable indicator of stress.

Objectives: The general objectives of this thesis are to investigate influencing factors on HCC, and to examine the potential of hair cortisol concentration as an indicator of long-term stress in cattle and pigs. Thus, the present studies aimed to (1) identify knowledge gaps in hair cortisol research, (2) evaluate the impact of animal-based, seasonal and hair-specific factors as well as contamination and elimination on HCC, and (3) investigate whether and when long-term increased systemic cortisol levels are reflected in elevated HCCs.

Animals, Material and Methods: Hairs were sampled from Holstein Friesian cattle, Landrace or Saddleback pigs and crossbreeds. The findings of the literature review (Study 1) identified potential animal-based, seasonal, hair-specific and stress-related factors on HCCs, which our experimental studies considered. To examine the impact of influencing factors (Study 2), a total of 614 animals were used. Hair samples were taken at different ages (newborn to adult), from different sexes and during both summer and winter. Variations by hair-specific factors were determined by studying black and white hair samples, varying body regions (neck/shoulder, back and tail tip) and different hair segments. In general, female animals were used. The effect of contamination on HCCs was examined in an *in vitro* study (Study 4) using hair samples from 12 cows and 12 sows. Samples were treated daily with urine, saliva, faeces or water for four weeks or remained untreated. To investigate long-term stress (Study 3), 34 cattle and 38 gilts were injected intramuscularly either with ACTH solution or saline every second day for four weeks. Natural and regrown hair samples were taken before and three times after the end of treatment, and hair segments were collected.

All the hair samples were shaved with electric clippers, washed twice with isopropanol and ground with a ball mill. Cortisol was detected by ELISA after extraction with methanol. Statistical analyses were performed using ANOVA and pairwise comparisons of the least square means by Tukey-Kramer tests with the MIXED procedure in SAS/STAT software.

Results: The results of Study 2 showed significantly higher HCCs in newborn calves than in young cattle, heifers and cows ($p < 0.001$). Likewise, 2-week-old piglets had higher HCCs than pigs aged 10 or 27 weeks and sows ($p < 0.001$). Sex had no effect on HCCs in pigs or cattle. In both species, HCCs were also significantly higher in samples obtained from the tail tip than from the shoulder, neck and back regions ($p < 0.001$), in black hair than in white hair ($p < 0.05$) and in distal hair than in proximal hair segments ($p < 0.001$). Season had an impact on HCC only in cattle, which exhibited higher levels in winter than in summer ($p < 0.001$). The results of Study 4 showed that contamination with urine caused a considerable concentration-dependent increase in HCCs in both species. Contamination with saliva and faeces also raised HCCs, but only in cattle (all $p < 0.05$). Treatment with water washed cortisol out from porcine hair but not from bovine hair. In cattle, repeated ACTH application (Study 3) revealed significantly higher HCCs after the end of treatment in natural hair (up to eight weeks, $p < 0.001$), regrown hair (up to four weeks, $p < 0.01$) and segmental hair (eight weeks, $p < 0.05$) than in the control animals. The highest HCCs were found four weeks after the end of treatment. In pigs, elevated HCCs were observed in both ACTH and control animals in all hair sample types after the application period, with no differences between treatments.

Conclusions: These results show that hair cortisol concentrations in pigs and cattle are affected by age, body region, hair colour, hair segment and season. There is first evidence that contamination of porcine and bovine hair with cortisol-containing body fluids, such as urine and saliva, may cause the incorporation of external cortisol into the hair shaft. Thus, when using HCC as a potential stress indicator, these influencing factors should be standardised and contamination effects should be avoided, such as by using the shave-reshave procedure, clean sampling regions and only the most proximal hair segments. The results also demonstrated that long-term stress by repeated activation of the HPA axis increases hair cortisol concentrations. In cattle, HCCs in different hair sample types reliably reflected the preceding period with increased systemic cortisol levels. In conclusion, the analysis of HCC appears to be a suitable method to evaluate long-term stress in cattle and pigs and can therefore be an important component in the assessment of animal welfare.

7 Zusammenfassung

Verfasser: Susen Heimbürge

Titel: Cortisolkonzentrationen in Haaren von Rind und Schwein: Einflussfaktoren und Eignung als Indikator für Langzeitstress

Institutionen: Institut für Tierhygiene und Öffentliches Veterinärwesen, Veterinärmedizinische Fakultät, Universität Leipzig

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Schlüsselwörter: Haarcortisol, Langzeitstress, Rind, Schwein, ACTH, Kontamination, Animal Welfare

Einleitung: Nutztiere sind aufgrund ihrer Haltungsbedingungen diversen Stressoren ausgesetzt, welche ihre physische und psychische Gesundheit beeinträchtigen können. Im Rahmen von Animal Welfare Monitoring besteht daher ein großes Interesse an der Entwicklung und dem Einsatz minimal-invasiver Methoden und tierbezogener Belastungsindikatoren. Haarcortisol-konzentrationen (HCCs) könnten hierfür ein vielversprechender, retrospektiver Stressindikator sein, da sie die durchschnittlichen systemischen Cortisolkonzentrationen der letzten Wochen bis Monate in nur einer Probe widerspiegeln. Bevor HCCs jedoch als zuverlässiger Indikator eingesetzt werden können, müssen potenzielle Einflussfaktoren darauf ermittelt werden.

Ziele: Das generelle Ziel der vorliegenden Arbeit ist die Untersuchung von Einflussfaktoren auf Haarcortisol und seine Eignung für die Beurteilung von Langzeitstress bei Rindern und Schweinen. Daher zielten die vorliegenden Studien darauf ab, (1) Wissenslücken in der Forschung zu identifizieren, (2) die Wirkung potenzieller Einfluss- und Störfaktoren zu evaluieren und (3) zu untersuchen, ob und wann erhöhte systemische Cortisollevel durch HCCs nachweisbar sind.

Tiere, Material und Methoden: Alle Haarproben wurde von Holstein-Rindern, Landrasse- und Sattelschweinen bzw. Kreuzungstieren entnommen. Aufgrund der Literaturrecherche (Studie 1) wurden potenzielle Einflussfaktoren identifiziert und in den nachfolgenden Studien untersucht. Für die Evaluierung nicht-stressbedingter Faktoren (Studie 2), wurden insgesamt 614 Tiere verwendet. Hierfür wurden Haarproben in verschiedenen Altersstufen (Neugeborene bis Erwachsene), von beiden Geschlechtern und während der Sommer- und Wintersaison entnommen. Außerdem wurden schwarze und weiße Haarproben, unterschiedliche Körperregionen (Nacken/Schulter, Rücken, Schwanzspitze) und verschiedene Haarsegmente untersucht. Der Einfluss von Kontaminationen auf HCCs wurde in einer *in-vitro*-Studie (Studie 4) mit Haarproben von 12 Kühen und 12 Sauen evaluiert. Diese Proben wurden für vier Wochen täglich mit Urin, Speichel, Kot oder Wasser behandelt oder blieben unbehandelt. Um die Wirkung von Langzeitstress auf HCCs zu untersuchen (Studie 3), wurde bei 34 Rindern und 38 Jungsauen über vier Wochen jeden zweiten Tag ACTH- oder Kochsalzlösung appliziert. Vor, sowie zu drei Zeitpunkten nach Ende der Behandlung, wurden native und neu gewachsene Haare sowie zusätzlich Haarsegmente

entnommen. Alle Haarproben wurden zweimal mit Isopropanol gewaschen, mit einer Kugelmühle gemahlen und Cortisol nach Extraktion mit Methanol mittels ELISA nachgewiesen. Die statistische Auswertung erfolgte mittels SAS/STAT-Software unter Anwendung von ANOVA und paarweisen Vergleichen durch Tukey-Kramer Tests.

Ergebnisse: Die Ergebnisse der Studie 2 zeigen signifikant höhere HCCs bei neugeborenen Kälbern im Vergleich zu Jungrindern, Färsen und Kühen ($p < 0,001$). Ebenso wiesen 2 Wochen alte Ferkel höhere HCCs auf als Schweine im Alter von 10 oder 27 Wochen oder Sauen ($p < 0,001$). Das Geschlecht hatte keinen Einfluss auf die HCCs beider Tierarten. Jedoch waren bei beiden Spezies die HCCs in Schwanzhaaren, im Vergleich zu den Schulter-, Nacken- und Rückenhaaren signifikant erhöht ($p < 0,001$), ebenso in schwarzen Haaren im Vergleich zu weißen Haaren ($p < 0,05$) und in distalen im Vergleich zu proximalen Haarsegmenten ($p < 0,001$). Außerdem wiesen Rinder im Winter höhere HCCs als im Sommer auf ($p < 0,001$). Die Ergebnisse der Studie 4 zeigen, dass die Kontamination mit Urin bei beiden Spezies eine konzentrationsabhängige Zunahme der HCCs bewirkt. Auch die Kontamination mit Speichel und Kot erhöhte die HCCs, jedoch nur bei Rindern (alle $p < 0,05$). Die Behandlung mit Wasser führte zu einer Auswaschung von Cortisol aus Haaren vom Schwein, jedoch nicht vom Rind. Die Ergebnisse der ACTH-Studie (Studie 3) zeigen signifikant erhöhte HCCs bei ACTH-Tieren zum Ende der Behandlung in nativen Haaren ($p < 0,001$), nachgewachsenen Haaren ($p < 0,01$) und in Haarsegmenten ($p < 0,05$). Die höchsten HCCs wurden innerhalb von vier Wochen nach Behandlungsende gefunden. Bei Schweinen wurde ein Anstieg der HCCs sowohl in ACTH- als auch in Kontrolltieren beobachtet ohne dass Unterschiede zwischen den Behandlungen auftraten.

Schlussfolgerungen: Die Ergebnisse zeigen, dass die Cortisolkonzentrationen im Haar bei Schweinen und Rindern durch Alter, Körperregion, Haarfarbe, Haarsegment und Jahreszeit beeinflusst werden. Es gibt erste Belege, dass die Kontamination von Schweine- und Rinderhaaren mit cortisolhaltigen Körperflüssigkeiten, wie Urin und Speichel, die Aufnahme von externem Cortisol in den Haarschaft verursacht. Bei der Verwendung von HCC als Stressindikator sollten daher diese Einflussfaktoren standardisiert und Kontaminationseffekte vermieden werden, z.B. durch die Verwendung von Aufwuchsproben oder nur proximaler Haarsegmente. Darüber hinaus konnte gezeigt werden, dass Langzeitstress durch wiederholte Aktivierung der HPA-Achse zu erhöhten Cortisolkonzentrationen im Haar führt. Bei Rindern zeigt sich dies in den HCCs verschiedener Haarprobentypen. Insgesamt erweist sich die Analyse von Cortisol im Haar als eine geeignete Methode zum Nachweis von Langzeitstress bei Rindern und Schweinen und könnte daher eine wichtige Komponente bei der Beurteilung von Animal Welfare sein.

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