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

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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.

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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

None.

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