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DETERMINATION OF HAIR CORTISOL CONCENTRATION AS A NON-INVASIVE METHOD FOR ASSESSING STRESS IN HORSES: ADVANTAGES, LIMITATIONS, AND THE NEED FOR STANDARDIZATION

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Abstract

The determination of cortisol concentration in hair is a promising and increasingly applied approach for evaluating long-term stress in animals, including horses. As a non-invasive, chemically stable, and easily handled biological material, hair offers clear methodological advantages over conventional matrices such as blood, saliva, or feces. Despite its growing use in stress research, the current literature reveals substantial gaps in the standardization of analytical procedures. These include inconsistencies in sampling site selection, sample preparation (including fragmentation), incubation parameters, and cortisol quantification methods. This paper reviews the methodological advantages and analytical challenges of using hair cortisol as a non-invasive biomarker of prolonged stress in horses, emphasizing its potential for welfare assessment. The review highlights the need for validated and harmonized protocols to enhance the reliability, reproducibility, and comparability of hair cortisol measurements across studies, thereby supporting its broader application in animal welfare research.

Keywords: hair cortisol concentration, non-invasive biomarker, equine welfare, chronic stress assessment, methodological standardization

The influence of stress on equine welfare

Recent years have witnessed a significant shift in public awareness and understanding of the psychological and emotional aspects of animal life. These changes have spurred an interest in animal welfare that extends beyond the traditional focus on livestock productivity. There is a growing number of initiatives aiming to provide animals with living conditions that best meet their natural needs. Stress and its impact on bodily functions is a particularly important consideration in the context of animal welfare research. Stress is defined as a physiological and behavioral response to stimuli that are perceived as a threat or a challenge and trigger adaptive mechanisms, thus enabling animals to acclimatize to unfavorable environmental conditions

and to cope with physical or psychological threats. These responses mobilize energy resources that are necessary for coping with challenges (Foreman and Ferlazzo, 1996; König von Borstel et al., 2017). Although stress is commonly associated only with negative effects, it is in fact a multifaceted and complex phenomenon. Not all types of stress are harmful, and in some cases, stress can be perceived as a neutral or even a positive condition that promotes adaptation (Trevisi and Bertoni, 2009).

In turn, distress is defined as a situation in which stress levels exceed an animal's adaptive capabilities and the evoked stress response provides no benefits, leading to a deterioration in its physical and psychological well-being. Chronic stress is one of the most harmful manifestations of this phenomenon, where prolonged exposure to adverse factors persistently activates bodily systems involved in the stress response. This type of stress can last weeks or even months, leading to the dysregulation of mechanisms responsible for the restoration of homeostasis, including the activation of relaxation responses and physiological and behavioral adaptation processes (Trevisi and Bertoni, 2009; Nejad et al., 2022). Chronic stress can be caused by prolonged exposure to the same type of stressors or their excessive number or frequency (Trevisi and Bertoni, 2009; Nejad et al., 2022).

The symptoms of chronic stress can manifest both directly and indirectly. Direct symptoms include an elevated body temperature, decreased energy levels, weight loss, reproductive disorders, anxiety, and suffering. Indirect symptoms involve hormonal, immunological, and metabolic changes (Trevisi and Bertoni, 2009; Budzyńska, 2014; Ataallahi et al., 2022; Nejad et al., 2022). In the long term, stress can lead to pre-pathological or even pathological conditions that pose a significant threat to the animal's health and life. Chronic stress not only increases susceptibility to disease, but it can also be a significant factor in the initiation and progression of various diseases (Trevisi and Bertoni, 2009; Nejad et al., 2022; Ataallahi et al., 2022).

In addition to physiological responses, chronic stress also exerts negative effects on animals' psychological well-being and behavior. The psychological symptoms of stress include increased fear, anxiety, and irritability, a decline in learning ability, and adaptive difficulties. In horses, chronic stress can lead to social withdrawal, decreased interest in their surroundings, and heightened response to environmental stimuli. In extreme cases, prolonged stress may lead to emotional dysregulation and increased susceptibility to depressive disorders, which has been documented in other animal species, including rodents and primates (McEwen and Wingfield, 2003; Valenchon et al., 2013).

Stereotypical or repetitive behaviors are one of the characteristic symptoms of chronic stress in horses. These invariant behaviors have no apparent goal or adaptive function, and they are not observed in feral horses living in natural habitats. The most common stereotypies in horses are windsucking, weaving, stall-walking, crib-biting, and wood chewing (Kowalski, 2005; Sarrafchi and Blokhuis, 2013). These behaviors are generally considered as indicators of decreased equine welfare resulting from environmental restrictions and the inability to engage in natural behaviors.

Chronic stress can also trigger aversive behaviors in horses, such as kicking, biting, rearing, head tossing, or bucking (Budzyńska, 2014). However, the absence of overt behavioral problems does not rule out chronic stress because some behaviors can be eliminated through training, thus masking the animal's true state (De Santis et al., 2017).

As highly alert animals that have evolved rapid defense responses to potential threats, horses are particularly susceptible to environmental stressors. Although horses' basic needs, such as access to food and protection against predators, have been fulfilled through domestication, the anthropogenic environment has introduced new adaptive challenges, including social isolation in stable boxes, transport, establishment of a social hierarchy when a new animal is introduced to a herd, training, injuries, and diseases (Werhahn et al., 2012; Schmidt et

al., 2010; Gardela et al., 2020; Flauger, 2011; König von Borstel et al., 2017; Deichsel et al., 2015; Medill et al., 2023; Berghold et al., 2007).

The fact that horses exhibit highly individual responses to stress should also be considered in the process of identifying the key stressors in this animal species. The nature and intensity of a perceived stressor are influenced by many factors, including the type and duration of the stress-inducing stimulus, its intensity, genetic factors, the animal's age, current physiological state, and previous experience (Trevisi and Bertoni, 2009; De Santis et al., 2017). These observations clearly indicate that chronic stress can be directly responsible for a significant decline in equine welfare (Trevisi and Bertoni, 2009; Nejad et al., 2022).

Cortisol as a physiological indicator of a stress response in animals

Cortisol is one of the key hormones involved in the body's physiological response to stress. This glucocorticoid hormone is synthesized in the zona fasciculata of the adrenal cortex. Cortisol has a relative long half-life (approx. 100 minutes), and the time course of cortisol's action is longer than that of other stress hormones, such as adrenaline and noradrenaline. In addition, cortisol can intensify and prolong the action of these hormones, thereby supporting adaptive metabolic changes under stress.

Physiological responses to stress are initiated by the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal (HPA) axis. The SNS triggers an immediate response by secreting adrenaline and noradrenaline, which increases the heart rate and blood pressure and mobilizes energy reserves to elicit the fight-or-flight response. The HPA axis is activated later than the SNS, and it is responsible for the body's response to prolonged or repeated stress. A stress-evoking stimulus triggers the secretion of the corticotropin-releasing hormone (CRH) by the hypothalamus, which stimulates the anterior pituitary gland to release the adrenocorticotropic hormone (ACTH). Having reached the adrenal glands, ACTH stimulates the production and secretion of glucocorticoids, mainly cortisol and corticosterone. Cortisol affects many tissues and organs because cortisol receptors are found in nearly all cells of the body (De Santis et al., 2017; Werner and Cole, 2021).

The mechanism by which cortisol penetrates hair has not been fully elucidated. It is believed that this lipophilic hormone reaches the hair shaft and the tissues surrounding the hair follicle by passive diffusion from the bloodstream. However, this observation has not been substantiated by empirical evidence (Heimbürge et al., 2019; Gardela et al., 2020; Saluti et al., 2022).

In animals, cortisol levels fluctuate under the influence of many biological and environmental factors, including sex. Mares, stallions, and geldings may respond differently to stressors due to differences in their hormonal and reproductive systems. Pregnancy is a special period during which cortisol levels increase in mares, and the highest concentration of this hormone in saliva is noted in the perinatal period (Aurich et al., 2015). However, research has shown that feral mares with foals or yearlings had higher cortisol levels in hair than females without dependent offspring (Medill et al., 2023). Stallions experience moderate chronic stress during the breeding season, not only due to the heightened demands of sexual activity but also the presence of other males in a herd (Aurich et al., 2015). Geldings are incapable of reproduction, but their cortisol levels have not been definitively established. Nowak et al. (2025) found no differences in the cortisol levels in feces of mares and geldings, whereas Zítek et al. (2025) reported higher hair cortisol concentration in geldings.

Diurnal and seasonal rhythms also affect cortisol levels. Salivary and serum cortisol concentration is usually highest in the morning (6–9 a.m.) and lowest in the evening (Lebelt et al., 1996; Cordero et al., 2012; Aurich et al., 2015). On a seasonal basis, both hair and serum cortisol levels increase between summer and fall, potentially helping the body to adapt to win-

ter conditions, such as decreased availability of food and exposure to thermal stress (Gardela et al., 2020; Cordero et al., 2012; Mazzola et al., 2021).

Age also plays a significant role in regulating cortisol levels. Neonates, including foals, have a higher concentration of this hormone in hair than older animals (Montillo et al., 2014; Heimbürge et al., 2019). Cortisol levels, both in serum and in hair, decrease with age, are relatively stable in adulthood, and increase once again in old age, when adaptive homeostasis declines (Cordero et al., 2012; Mazzola et al., 2021). Daily fluctuations in serum cortisol concentration are reduced with age, which could be attributed to a decrease in the activity of adrenal glands (Cordero et al., 2012).

Cortisol levels are also influenced by housing conditions. Different housing systems (confined, semi-confined, and free-range) regulate social interactions, access to space and food. Stress levels, measured with hair cortisol, were found to be lowest in horses kept in a semi-confined system and highest in animals with unrestricted access to a paddock (Mazzola et al., 2021). Horses deprived of contact with other members of their species also demonstrated higher fecal cortisol levels (Yarnell et al., 2015). Changes in the housing system can initially provoke a rise in both hair and serum cortisol concentration, but animals gradually adapt to new conditions (Werner and Cole, 2021; Aurich et al., 2015).

Advantages of hair cortisol measurement compared to other biological matrices

Unlike blood, saliva, feces, urine, milk, tears, or hooves (Ataallahi et al., 2022; Sikorska et al., 2023), hair represents a unique biological matrix that supports reliable and long-term evaluations of chronic stress. Unlike other materials that reflect only transient or short-term hormonal fluctuations, hair stores integrated information about the animal's physiological state over extended periods – weeks, months, or even years (Greff et al., 2019). When the average hair growth rate is known, segmental analysis allows researchers to reconstruct a retrospective timeline of stress exposure and to link specific environmental events with physiological responses. For instance, equine tail hair grows at approximately 20 mm per month (Burnik Šturm et al., 2015; Duran et al., 2017; Medill et al., 2023), enabling month-by-month tracking of chronic stress patterns in individual animals.

Hair cortisol determination offers several key advantages over conventional matrices. It is entirely non-invasive, eliminating the stress, discomfort, and handling bias associated with repeated blood or saliva collection, thus improving animal welfare and ensuring compliance with ethical standards of animal research (Stalder and Kirschbaum, 2012). Sampling is simple, quick, and inexpensive, requiring neither specialized equipment nor trained personnel, which makes it suitable for large-scale and field studies. Furthermore, hair is chemically stable, resistant to microbial degradation, and extremely easy to store and transport compared with biological fluids such as blood, saliva, milk, or urine, which require refrigeration and rapid analysis. Hair can be stored at room temperature for months without loss of hormone integrity, or virtually indefinitely at −20°C (Russel et al., 2012; Saluti et al., 2022). The exceptional stability of cortisol in hair is illustrated by analyses of ancient mummified remains, where cortisol profiles remained consistent with modern reference values (Webb et al., 2010).

Another major advantage is the possibility of retrospective and segmental analysis, which allows the reconstruction of physiological history in individual animals – an ability unmatched by any other matrix. The method also demonstrates positive correlations with cortisol concentrations in saliva, urine, and feces, providing strong validation of its physiological relevance (Meyer and Novak, 2012). Because hair integrates hormonal secretion over time, it smooths short-term fluctuations and yields a robust indicator of chronic HPA axis activity,

minimizing the confounding influence of momentary stressors that can distort results in blood or saliva tests.

Collectively, these features make hair cortisol analysis a powerful, welfare-friendly, and methodologically convenient tool for assessing long-term stress in horses and other animals. Its non-invasiveness, sample stability, and retrospective potential clearly outweigh the remaining methodological challenges related to standardization. As such, hair cortisol should be regarded as one of the most promising and versatile biomarkers for evaluating animal welfare in both research and applied settings.

Table 1 summarizes the comparative characteristics of biological matrices commonly used for cortisol determination. Among these, hair clearly stands out as the most welfare-friendly, stable, and informative matrix, offering unique advantages for assessing long-term stress exposure in animals.

Table 1. Comparison of biological matrices used for cortisol determination in animals

Biological matrix	Sampling invasiveness	Reflects time scale of stress	Sample stability and storage	Analytical reliability	Main advantages	Main limitations	References
Blood (serum, plasma)	Highly invasive – requires venipuncture and trained personnel	Acute (minutes–hours)	Poor – rapid degradation, requires refrigeration	High (if handled correctly)	Direct measurement of circulating cortisol; well-standardized assays	Sampling induces stress; strong diurnal fluctuations; unsuitable for chronic stress	Foreman and Ferlazzo, 1996; Nejad et al., 2022
Saliva	Minimally invasive but requires handling	Short-term (minutes–hours)	Moderate – must be refrigerated; possible bacterial degradation	High, but sensitive to contamination	Easy to collect repeatedly; reflects free cortisol fraction	Collection itself may cause stress; affected by feeding, circadian rhythm	Lebelt et al., 1996; Aurich et al., 2015
Feces	Non-invasive	Intermediate (hours–days)	Moderate – requires drying or freezing	Good when standardized	Reflects metabolized cortisol; suitable for group-level assessment	Delayed reflection of stress; environmental degradation; variable metabolism	Nowak et al., 2025; Nejad et al., 2022
Urine	Minimally invasive (requires collection control)	Short-term (hours–day)	Limited – must be frozen; prone to bacterial contamination	Good	Integrates short-term cortisol secretion	Requires controlled collection; affected by hydration and diuresis	Ataallahi et al., 2022
Milk	Moderately invasive (requires milking)	Short-term (hours–days)	Moderate – requires refrigeration	Moderate	Useful in dairy species; easy routine sampling	Species-limited; not applicable to all animals	Comin et al., 2014; Sikorska et al., 2023
Tears	Minimally invasive (ophthalmic collection)	Acute (minutes–hours)	Poor – small volume, unstable	Moderate	Useful in clinical diagnosis	Stress-inducing sampling; technically demanding	Hart et al., 2016
Hooves	Non-invasive	Long-term (weeks–months)	Excellent	Limited data	Retrospective potential similar to hair	Difficult sampling and processing; less validated	Keogh et al., 2023
Hair	Non-invasive; painless; no restraint needed	Long-term (weeks–months–years)	Excellent – stable at room temperature or frozen	High (validated vs. other matrices)	Welfare-friendly; retrospective analysis possible; easy transport and storage; low cost	Requires methodological standardization (sampling site, fragmentation, extraction)	Greff et al., 2019; Meyer and Novak 2012; Saluti et al., 2022; Heimbürge et al., 2019

Technical considerations in hair sample preparation and cortisol extraction

Although the methodological basis of hair cortisol analysis is relatively straightforward, its accuracy and comparability depend on the consistent application of standardized sampling and preparation procedures. The following sections summarize the main technical factors that influence measurement reliability and highlight areas where methodological harmonization is still needed.

Hair analysis is a well-established scientific tool in various research disciplines, including studies assessing exposure to drugs, environmental contaminants, nutrients, anabolic and sex steroids, and other hormones (Accorsi et al., 2008; Greff et al., 2019). In recent years, special attention has been paid to hair cortisol as a valuable indicator of chronic stress in animals. Nevertheless, despite its increasing use, substantial differences in experimental protocols persist between studies. These discrepancies concern the sampling site and method, sample processing techniques, incubation parameters, and analytical procedures, all of which may significantly influence the comparability and interpretation of results.

Hair sampling site and sampling procedure

There are many techniques for sampling hair for analysis, depending on the type of hair (coat, mane, or tail) and the purpose of the analysis. Hair should be clipped as close to the skin as possible to obtain a representative sample (Nejad et al., 2022). Hair shaving with an electric shaver or a disposable razor is most widely recommended in the literature. This method provides high accuracy by allowing the collection of samples of uniform length at a minimal distance from the skin, which is an important consideration in analyses of recent hair growth. Hair can also be cut with scissors, but this method is not as accurate, and the collected samples may differ in length (Nejad et al., 2022).

In many animal species, including horses, coat hair grows to a certain length and then stops, after which the hair is shed and replaced by newly growing hair. The hair life cycle consists of three main stages: the anagen stage (active growth), the catagen stage (transitional growth), and the telogen stage (resting phase). Cortisol can be incorporated into the hair structure only during the anagen stage. The growth of coat hair is not synchronous, and a sample collected by a standard method contains hair in which cortisol was deposited in different time intervals (Meyer and Novak, 2012; Heimbürge et al., 2019). The shave–reshave technique is particularly useful in this context because it supports accurate cortisol determination in coat hair grown in specific periods of time. In this approach, a small, marked patch of skin is shaved completely (usually a square with a side length of several to more than ten centimeters), marking the starting point for hair regrowth in the examined period. At the end of that period, the same area is shaved again, and the collected sample reflects cortisol deposition in hair that regrew between the first and the second sampling, thus markedly improving control over the analyzed time interval. In this method, the sampling site has to be accurately marked, and hair has to be shaved with precision during the second sampling to prevent contamination with hair growing outside the sampling site (Meyer and Novak, 2012; Heimbürge et al., 2019; Nejad et al., 2022). The shave–reshave technique has been applied in studies of rhesus monkeys (Davenport et al., 2008), sheep (Salaberger et al., 2016), and horses (Gardela et al., 2020; Mazzola et al., 2021; Olvera-Maneu et al., 2021).

Mane and tail hair is usually collected with scissors by precisely incising a strand of hair at the collection site. Mane and tail hair is longer, and it can be segmented into fragments corresponding to specific weeks or months, which is particularly useful for analyzing long-term changes in cortisol levels. In some studies (Duran et al., 2017; Jolivald et al., 2022; Medill et al., 2023), samples were obtained by plucking hair with follicles, either manually or

with a comb. In horses, hair can also be sampled during mane pulling by removing the follicle and dividing the hair into segments that correspond to specific time intervals. Hair segments closer to the follicle represent the most recent growth, whereas more distant fragments correspond to earlier stages of the hair growth cycle (Meyer and Novak, 2012). The main advantage of this method is that it allows for a detailed determination of changes in cortisol levels, including in the most recent regrowth which is often lost when hair is clipped with scissors. However, samples should be collected with great caution to prevent contamination with follicle fragments that may constitute an independent source of cortisol (Russel et al., 2012).

Meyer and Novak (2012) found that human hair follicles stored *in vitro* are able to synthesize not only cortisol, but also CRH, corticotropin, and their receptors, creating a local hormonal regulation system similar to HPA axis. A negative feedback mechanism was also observed, whereby cortisol inhibited CRH production. Although cortisol is not rapidly incorporated into the hair structure, these results indicate that local cortisol production can partly affect the total concentration of this hormone in hair (Russel et al., 2012).

A number of methodological factors that could affect the reliability and comparability of results should be considered during hair sampling. One of them is the risk of sample contamination with cortisol from external sources. Hair can be contaminated with feces or urine, which are also used as matrices for the determination of cortisol levels. In addition, cortisol can also be present in skin secretions such as sweat or sebum on the surface of hair (Greff et al., 2019). Therefore, hair should be sampled in sites that are least exposed to contact with these contaminants. For example, tail hair should be collected from the base of the tail, where the risk of contamination with feces and urine is minimal (Nejad et al., 2022).

Interesting results were reported by Salaberger et al. (2016) who found that mechanical factors affected cortisol concentration in sheep wool. The wool coat was combed twice daily for 21 days, and cortisol levels were higher in combed than uncombed wool, which implies that intensive combing can lead to local alterations in cortisol concentration (Salaberger et al., 2016). Daily grooming, such as brushing, can elicit similar effects in horses, particularly those used for recreation and sports. Therefore, when planning research, investigators should ensure that the analyzed body area is not subjected to intensive care for a specified period of time or that less exposed sites are selected. However, this is the only study to report on local changes in cortisol levels, which warrants further investigation.

Some researchers found that cortisol concentration tends to decrease gradually with distance from the hair base. This observation was attributed to external factors, such as the use of shampoo, contact with water, and UV radiation. However, these studies were conducted on humans who, unlike horses, regularly wash hair and use shampoo. Therefore, it can be assumed that cortisol levels in horse hair are not significantly influenced by contact with water and detergents. In turn, exposure to UV radiation and sunlight can degrade hair cortisol, which is known as a "wash-out" effect. Wester et al. (2016) reported that hair cortisol concentration decreased by 32% under exposure to sunlight and by as much as 50% under exposure to artificial UV light. Cortisol levels decreased by 54% after 40 hours of sunlight exposure relative to hair samples stored in darkness. The wash-out effect can confound the results, especially in analyses of stress levels in more distant periods of the animal's life. Therefore, the wash-out effect should be considered during experimental design and data interpretation, and samples should be stored in adequate conditions, away from sunlight (Meyer and Novak, 2012).

Cortisol concentration can also differ across sampling sites. Duran et al. (2017) reported higher cortisol levels in the mane than in the tail of Haflinger mares, although the observed differences were not significant in yearlings. However, cortisol concentration tended to be lower in the tail. In turn, Sauveroche et al. (2020) found a positive correlation between cortisol levels in mane hair and the hair coat on the withers, but they concluded that samples

should be collected from the mane due to smaller individual differences in hair length and thickness, compared to body hair. Lellákova et al. (2022) reported no significant differences between hair sampled from the forelock, the withers, and the tail, and they observed the lowest cortisol concentration in hair from the chest region. The observed differences in hair cortisol levels in various parts of the body could be attributed to specific stages of the hair growth cycle (as demonstrated in the common marmoset), possible contamination with excreta, exposure to environmental factors, grooming, or differences in skin blood flow (Heimbürge et al., 2019; Lellákova et al., 2022). Lellákova et al. (2022) and Duran et al. (2017) emphasized that hair samples for cortisol analysis should be collected from the same region of the body to ensure the comparability of results.

Procedure of cleaning hair samples

As previously mentioned, hair samples can contain various environmental contaminants. Samples are usually washed before analysis to remove potential contaminants. However, this preparatory step may be omitted, especially in analyses of total cortisol levels, including fractions from skin secretions such as sweat and sebum. Hair samples are usually washed with isopropanol, although methanol (Duran et al., 2017; Saluti et al., 2022) and distilled water (Mazzola et al., 2021) have also been used.

Davenport et al. (2006) evaluated the effectiveness of various hair washing methods. Preliminary tests involving dichloromethane, water, phosphate buffer, and aqueous solutions of sodium dodecyl sulfate – compounds that are widely used in doping control tests – yielded unreliable results in cortisol analyses. Next, the effectiveness of isopropanol and methanol and a different number of wash cycles (one, two, and three wash cycles lasting 3 minutes each) were compared. Methanol removed increasing quantities of cortisol in subsequent wash cycles, which suggests that this compound penetrates into the hair shaft and extracts internally deposited cortisol. In turn, when hair was washed with isopropanol, the amount of extracted cortisol stabilized after the second wash, indicating that isopropanol removed only surface contamination without penetrating the hair shaft. These results confirm that isopropanol is the ideal medium for washing hair samples before cortisol extraction. Washed samples should be left to dry completely under controlled conditions.

Hair fragmentation methods

The structural specificity of the processed material has to be considered and the appropriate fragmentation method has to be selected in the next stage of sample preparation for analysis. Efficient cortisol extraction requires ensuring the maximum contact area between the hair medulla and the solvent, such as methanol (Greff et al., 2019). According to the literature, hair samples are usually pulverized or cut into smaller fragments before analysis. Hair is usually pulverized in a bead mill or a bead homogenizer, and it is cut into fragments with surgical scissors or a scalpel for greater precision. Hair is usually cut into segments with a length of 1–4 mm (Koren et al., 2002; Accorsi et al., 2008; Fourie et al., 2016; Casal et al., 2017), although in some studies, the size of hair fragments has been described as shorter than 5 mm (Salaberger et al., 2016). In many studies, the size of hair fragments is not indicated or described as “small pieces” (Sauvé et al., 2007; Gonzalez et al., 2019). The absence of a standardized fragmentation procedure makes the results across studies difficult to compare and undermines their reproducibility.

In addition, a portion of the sample is lost when hair is cut with scissors, and this method requires considerable precision and time, which limits its effectiveness. Despite these limitations, scissors are a useful tool when advanced equipment is not available. Comparative

studies have demonstrated that cortisol concentration was higher in pulverized than in cut samples (Davenport et al., 2006; Burnett et al., 2014). In pulverized samples, a larger proportion of material is exposed to the solvent, thus enhancing cortisol extraction. In turn, Stalder et al. (2012) reported a strong correlation between cortisol levels in pulverized and non-pulverized samples, highlighting the need for further investigation. It has also been hypothesized that hair grinding can lead to the thermal degradation of proteins and steroids, including cortisol (Slominski et al., 2015). However, this problem can be minimized through cryogenic grinding (cryomilling) with liquid nitrogen (Greff et al., 2019).

The most effective hair fragmentation method cannot be clearly identified based on the available data, indicating the necessity for further research on optimizing the preparation of hair samples for cortisol analysis.

The influence of incubation parameters on cortisol extraction efficiency

One of the key issues is the time of hair sample incubation, which is one of the most diverse and least standardized parameters in the cortisol extraction procedure. In most studies, hair samples were incubated for 18 to 24 hours (Sharma et al., 2019; Gardela et al., 2020; Heimbürge et al., 2019; Jolivald et al., 2022). However, some researchers have described incubation time as "overnight", without specifying its precise duration (Sauvé et al., 2007; Gonzalez et al., 2019; Sauveroche et al., 2020; van der Laan et al., 2022). In the work of Karlén et al. (2011), the minimum incubation time was 10 hours, whereas Skoluda et al. (2012) incubated hair samples for only 45 minutes. In contrast, Salaberger et al. (2016) described an incubation period of 72 hours. These discrepancies point to the lack of scientific consensus on the optimal incubation time, which was one of the motivations for undertaking this study.

In the standard procedure, cortisol is extracted from 10 mg of washed and weighed hair with the addition of 1–2 mL of methanol. The sample is usually incubated at a temperature of around 50°C, with constant stirring on a rocking platform or a shaker (Greff et al., 2019). However, in some studies, hair samples were incubated at 30°C (Sharma et al., 2019; Gardela et al., 2020), 37°C (Comin et al., 2014; Casal et al., 2017), and room temperature (Heimbürge et al., 2019; van der Laan et al., 2022).

Ultrasound exposure is an interesting alternative in the incubation process. Sonication is usually a preliminary step that is followed by standard incubation. Fourie et al. (2016) incubated baboon hair in a sonicating water bath to determine the effect of different incubation times (0, 60, 120, 240, and 360 minutes) on cortisol extraction efficiency. After sonication, all samples were incubated on a plate shaker for 24 hours. Samples sonicated for 240 minutes yielded the largest amount of cortisol, and longer exposure did not improve extraction efficiency. Sonicated samples yielded 1.5 times more cortisol than samples incubated at room temperature.

In other studies, hair samples were exposed to ultrasound for shorter periods of time. For example, Mazzola et al. (2021) incubated samples for 12 hours after 30 minutes of sonication. A similar approach was adopted by Koren et al. (2002), van Uum et al. (2008), and Burnett et al. (2014), but in these studies, hair samples were incubated overnight.

Methanol is most widely used for cortisol extraction, but other solvents have also been described in the literature. Crimele et al. (2000) used Sorensen's phosphate buffer, and other authors applied acetone as an alternative solvent. Despite the above, methanol is still the solvent of choice because it causes hair swelling by penetrating into the hair shaft, which leads to the solubilization of steroid hormones, including cortisol (Meyer and Novak, 2012).

However, a single incubation in methanol extracts only 40–60% of the total cortisol present in the sample. A four-stage procedure involving the intermittent use of methanol and acetone (15 h at 52°C and 5 min at room temperature, repeated twice) was proposed to in-

crease cortisol extraction efficiency to 98–100% (Slominski et al., 2015; Greff et al., 2019). Duran et al. (2017) developed an alternative approach, in which hair samples were subjected to three consecutive methanol extractions. After each extraction, the samples were centrifuged and the supernatant was collected, achieving more than 98% total cortisol recovery. A similar method was used by Medill et al. (2023).

Methods for analyzing cortisol concentration

Cortisol concentration in biological materials, including hair, can be determined using various analytical techniques. The most popular methods include immunoenzymatic tests (ELISA, EIA), mass spectrometry (LC-MS/MS, GC/MS), and radioimmunoassays (RIA). Commercial immunoenzymatic tests, in particular the enzyme-linked immunosorbent assay (ELISA), are most widely applied to determine cortisol levels. The ELISA is a popular test because it is relatively inexpensive, simple to perform, sufficiently sensitive, does not require specialist equipment, and can be applied to analyze multiple samples simultaneously.

However, ELISA can exhibit cross-reactivity with other steroids, such as cortisone and progesterone, which can lead to the overestimation of cortisol concentration in a sample. Therefore, kit-specific correction factors should be applied to ensure the reliability of the results (Greff et al., 2019). Mass spectrometry, especially when combined with liquid chromatography (LC-MS/MS), is also a valid alternative to immunoenzymatic tests, as it is characterized by very high sensitivity and specificity. This technique allows unambiguous cortisol detection without the risk of cross-reactivity. Although studies have demonstrated a correlation between ELISA and LC-MS/MS results, this correlation is low when absolute values are compared (Slominski et al., 2015; Nejad et al., 2022).

The RIA technique, once widely applied, is characterized by high sensitivity and high specificity for cortisol. However, this method requires radioactive isotopes and strict occupational safety methods, which has led to a gradual decline in its popularity (Nejad et al., 2022).

The diversity of analytical methods presents a significant challenge for advancing research on cortisol levels in hair samples. The observed discrepancies in analytical procedures, including hair sampling, sample preparation, incubation conditions, and methods of cortisol detection and quantification, highlight the absence of uniform and widely accepted procedural standards. Therefore, further research is needed to develop cohesive and validated analytical protocols. The standardization of analytical methodology would not only enhance the reliability and comparability of results, but would also improve the quality and consistency of research on chronic stress in humans and animals. Despite certain methodological challenges, the advantages of hair cortisol analysis clearly outweigh its limitations, especially in studies of animal welfare where non-invasiveness and long-term stress assessment are crucial.

Conclusions

Stress is an inherent part of animal life, and horses – being a particularly sensitive species – react strongly to stress-inducing stimuli. Cortisol is the primary hormone that participates in the stress response by mobilizing physiological systems to cope with the challenge. However, chronically elevated cortisol levels can result in adverse health effects and behavioral changes.

Various biological matrices can be used to determine cortisol concentration, including blood, saliva, feces, urine, milk, hooves, and hair. Conventional detection methods are invasive and may induce stress in animals; therefore, hair is increasingly recognized as an effective alternative matrix for assessing the effects of long-term stress exposure. Hair is easy to sample, resistant to degradation, and remains stable during storage.

In equine research, hair is usually collected from the tail, which simplifies the sampling procedure and minimizes contamination. The collected samples are cleaned with isopropanol, dried, and fragmented by cutting or grinding. Pulverization improves cortisol extraction efficiency by increasing the area of contact with the solvent, but it can lead to the thermal degradation of cortisol. This risk can be minimized through cryogenic grinding (cryomilling).

The incubation of biological material in methanol is a key step in cortisol extraction. The incubation times provided in the literature vary considerably, from several dozen minutes to several dozen hours. In some studies, incubation was enhanced through sonication to increase extraction efficiency. Other solvents have also been used, but methanol remains the solvent of choice as it facilitates cortisol diffusion from the hair shaft.

Immunoenzymatic assays, such as ELISA, are most widely used to determine cortisol levels. These tests are most commonly employed as they are relatively inexpensive, simple to perform, and suitable for analyzing multiple samples simultaneously. The main limitation of this technique is the risk of cross-reactivity with other steroids, which can lead to overestimation of the results. Spectrometric methods, in particular LC-MS/MS, provide greater specificity but require more advanced equipment. Radioimmunoassays are highly sensitive, but their popularity has declined due to occupational safety concerns.

The diversity of analytical methods and the lack of standardized techniques for sampling, preparation, and analysis of hair samples highlight the need for further research. The development of uniform procedures would improve the reliability and comparability of results across research centers. While standardization remains a challenge, the methodological simplicity, sample stability, and welfare-friendly nature of hair sampling make this approach uniquely suited for large-scale and longitudinal studies in equine science. Considering its non-invasive nature and long-term perspective, hair cortisol analysis should be regarded as one of the most promising tools for welfare assessment in horses, complementing and in many cases surpassing traditional biological matrices.

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