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Hair Cortisol: A Biomarker of Chronic Stress in Animals and its Association with Reproduction

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ABSTRACT

Hair cortisol concentration (HCC) has been used to assess the retrospective measurement of the hypothalamic-pituitary-adrenal (HPA) axis over a long period of time. HCC reflects HPA axis activity over a long period (months to years) depending on hair growth rate and length of hair measured. HCC is being used as a chronic stress biomarker in order to check the well-being of domestic animals (cattle, horses, dogs, pigs), wild animals, captive animals, human trauma victims, and post-traumatic stress disorders (PTSD) as it provides an innovative approach to measure chronic HPA activity retrospectively over months without being affected by short term acute stressors and diurnal patterns. Stress is a significant factor affecting animal fertility. Activation of HPA axis further activates the hypothalamic–pituitary–gonadal axis (HPG) i.e., glucocorticoids are produced causing apoptosis of Leydig cells and decreased testosterone levels in males. In females, increased cortisol levels can suppress the tonic secretion of Leutinizing hormone (LH) which might hamper ovulation. Thus, hair cortisol analysis for assessing the HPA-HPG axis can be an important tool for animal welfare.

Introduction

According to Hans Selye, stress is the body's non-specific response to any stressful stimuli. Cannon coined the concept of homeostasis, which is based on *milieu intérieur* (internal medium), a term given by Claude Bernard. Homeostasis involves the maintenance of all physiological systems in the state of dynamic equilibrium. According to Selye, General Adaptation Syndrome is characterized by three stages: (1) alarm phase- the period which indicates the presence of a stressor (2) resistance phase- disappearance of acute manifestations; and (3) exhaustion phase- the period when energy reserves are exhausted (Selye et al.,1936). Stress particularly chronic stress has a negative impact on animal health. The neuroendocrine axis hypothalamic-pituitary-adrenal axis (HPA) gets activated under stress conditions, leading to glucocorticoid secretion (cortisol).

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Measurement of cortisol

Cortisol assessment can be done in various samples, e.g., blood, urine, saliva, and feces (Table 1). The properties of various types of samples used for cortisol assessment have been mentioned in Table 1. Serum and saliva are mostly used to measure cortisol levels, although according to circadian rhythm, they are subject to change. Cortisol levels are the highest during early morning followed by a gradual decrease. Total serum cortisol is found in two forms, bound and free. However, salivary cortisol is known to be the free form and is a biologically active form collected by the non-invasive method in comparison to blood. Estimation of cortisol in blood being the most invasive approach can lead to variation in test results due to elevations in cortisol caused by the fear of venepuncture (Weckesser et al., 2014). Most of the studies reveal a high correlation between free cortisol levels in blood and saliva. Levine et al., (2007) reported the enzymatic conversion of 30% of cortisol to cortisone in saliva compared to plasma. In urine, only 1% of free blood cortisol is excreted. Urinary cortisol is affected by renal conditions like chronic renal failure. 24 h measurement of cortisol can be done in urine samples (Burch et al., 1982). Along with urinary cortisol, cortisol metabolites can also be assessed both in feces and urine which can be estimated in 24 h.

Table 1: Characteristics of samples used for cortisol assessment

Characteristics	Serum	Saliva	Urine	Hair
Invasiveness	High	Low	Moderate	Not much
Stress of sampling	High	High	High	Low
Measurement of cortisol	Single point	Single point	12-24 h integral exposure	Months to years
Available form	Total cortisol measured (free + bound form)	Free form measured	Free form measured	Free form measured

Techniques used for cortisol estimation

Various assays are used to detect cortisol levels radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and liquid chromatography coupled mass spectrophotometry (LC-MS/ MS). In the initial days LC-MS was used for HCC analysis, but nowadays ELISA has gained popularity. LC-MS can detect cortisol even in minute quantities ie 0.03 ng/mg of hair. ELISA on the other hand gives higher cortisol values besides LC-MS has more precision. There is limitation with ELISA that it has crossrectivity with progesterone (7.2%) and cortisone (6.2%). This 6.2% cross reactivity with cortisol antibody can increase cortisol with 1% which is still considered less (Slominski et al., 2015). RIA is very sensitive it can detect 100% cortisol, and the detection limit is 0.26 pg/mg of hair. The use of radioactive substances causes health hazards. Therefore now a days this technique is rarely used (Ghassemi Nejad et al., 2022).

Cortisol quantification in hair

Analysis of hair was done for forensic studies to check for the deposition of toxins and drugs in hairs and fingernails (Sachs et al., 1997). Various anabolic steroid hormones were detected in hair in a doping scandal in 1988 (Gaillard et al., 2000), and further estimation of endogenously produced hormones was also started. Cirimele et al. (2000) estimated glucocorticoids from human hair for the first time. Koren et al., (2002) in their initial studies estimated hair cortisol concentration (HCC) in wild hyraxes. After hair collection of 7-20 mg, methanol-assisted cortisol extraction from the hair was done. Using a modified salivary ELISA, the analysis was performed. A significant positive correlation between concentration of hair cortisol and the social hierarchy of wild hyraxes was determined. Higher concentration of cortisol was also related to greater dominance in the socially dominant group of wild hyraxes (Koren et al., 2008).

Davenport et al. (2008) quantified cortisol levels in Rhesus monkey hair and it was found strong correlation between salivary and hair cortisol concentration. Free cortisol is incorporated into hair as found in saliva. Both saliva and hair cortisol can be affected by adrenocortical secretion and circulating corticosteroid-binding globulin. Sauve et al. (2007) detected hair cortisol in non-obese humans using the ELISA protocol. With a median value of 46.1 pg/mg, a reference range for hair cortisol was substantiated, i.e., 1.7-153.2 pg/mg. Hair cortisol concentrations were determined and compared with cortisol concentrations of saliva, serum, and urine samples using 24hr collection. Although hair cortisol was not found to be significantly correlated with serum and salivary cortisol, there was a positive correlation between hair and 24 hr urine cortisol concentration. Davenport et al. (2008) reported appreciable correlation between hair and saliva cortisol levels of rhesus macaques. Bennett and Hayssen (2010) correlated hair and salivary cortisol levels in dogs. Estimation of cortisol deposited in hair is gaining popularity these days, as it is a promising biomarker for HPA activation (Wright et al., 2015). Hair cortisol is being used as a chronic stress biomarker to check well-being of human trauma victims (Karlén et al., 2011), post-traumatic stress disorders (PTSD) (Steudte et al., 2011), wild animals (Mastromonaco et al., 2014), captive animals (Ashley et al., 2011, Dettmer et al., 2014) and lab animals (Scorrano et al., 2014).

Cortisol deposition in hair

At the rate of 1 cm/month, hair grows in different stages, i.e., (i) anagen (active growth), (ii) catagen (transition stage), (iii) telogen (resting stage). Therefore a segment of 1 cm gives cortisol production from the previous month (Wennig et al., 2000). It provides a window to the past and gives the researcher a chance to examine previous cortisol concentration (Pereg et al., 2011). About 10 mg hair/ segment can be used for analysis. Thus, only a small amount of sample is required. There are several mechanisms by which cortisol enters hair. The most common hypothesis is complex multicompartmental models which involve the incorporation of a drug in the hair (Boumba et al., 2006). Through passive diffusion, cortisol enters hair at the hair shaft medulla from blood. The cortisol is deposited in free form (Pragst and Balikova, 2006). After emerging from the scalp, the growing shaft is coated with sebum along with sweat from the sebaceous gland and eccrine glands. Previous studies where human subjects were administered (IV) radiolabeled cortisol showed that cortisol can diffuse into sebum and sweat from blood (Cook et al., 1964). As a result, cortisol might be deposited on the outer surface of the hair shaft from aforementioned fluids. Whether this cortisol gets incorporated in the hair shaft or not is not yet clear. At the time of hair sampling, the stage of growth should also be taken into account. To ensure the collection of sufficient hairs in the growing stage, the "shave-reshave" method might be used. Re-grown hair sample is collected by reshaving a specific area which has been shaved earlier at a certain period of time.

Studies of hair cortisol in different species

Cattle

In cattle, the hair growth rate is not well defined. Dairy cows have an average growth rate of 0.04 ± 0.05 mm hair/day at the hip region, with a body average growth rate of 6–10 mm hair/month (Schwertl et al., 2003). As compared to other areas of body, tail switch as a significantly higher growth rate (10x) in lactating dairy cows, while hair in shoulder and hip area grow at a similar rate (Burnett et al., 2014). Fisher et al., (1985) reported similar results in beef cattle. Various factors like nutrient deficiency (Martin et al., 1969), photoperiod (Johnson et al., 1981), and temperature (Berman et al., 1961; Hayman et al., 1961) affect hair growth rate in cattle. Thus, hair cortisol concentration is majorly impacted by the growth rate of hair, and positively correlates with hair length. Moya et al., (2013) reported HCC in bulls ranging from 0.30 and 5.31 pg/mg of hair. As compared to the top line or hip, higher cortisol concentration was observed in the tail hair (Cerri et al., 2012). Burnett et al. (2015) reported in their findings that white hair cortisol concentrations were lower in the hip or top line as compared to tail in the case of lactating dairy cows. This is due to the increased growth of hair in the tail and heightened cortisol concentration (Moya et al., 2013). The breed is also an influencing factor in the hair cortisol concentration is influenced. As compared to Montbeliarde and Swedish Red crossbred heifers, Holstein-Friesian cows had higher hair cortisol concentrations (Peric et al., 2013). Holstein cows and heifers also had higher HCC as compared to Busha heifers and cows (Nedić et al., 2017), which might be due to higher milk yield and differences in production systems resulting in different metabolic demands and resultant stress. UV irradiation might be another factor contributing towards higher hair cortisol concentrations of Holstein cows compared to Busha cows (Wester et al., 2016), which might be the reason for elevated cortisol concentration in parts of hair closer to skin (proximal), as compared to the parts farther from the skin (distal) in Busha cows dwelling in mountain pastures (Nedić et al., 2017). Tallo-Parra et al. (2015) found that black hair from the same cow had higher HCC as compared to the white hair. A study on heat-stressed lactating Holstein cows showed that hair cortisol concentrations did not vary significantly in relation to the hair color, but as compared to the white coat colors, black coats had higher cortisol values. Therefore, the coat colour of the animal might have impacted the estimated cortisol levels. Thus, it was established that Holstein cows having white coat colour have a higher resistance to heat stress (Ghassemi et al., 2017). A study conducted on Holstein and Busha breeds (Nedić et al., 2017) found that hair colour had no effect on hair cortisol levels. In multiparous cows, hair cortisol values were slightly higher (Burnett et al., 2015). Therefore, more research is required to study the effect of hair colour on cortisol levels (Table 2).

Table 2: Studies of hair cortisol in cattle

Species/ breed	No of animals	Reference	Method	Clinical findings
Holstein-Friesian	44	González-de-la-Vara et al., 2011	RIAThe	• ACTH group had higher HCC compared to saline and control. • HCC is higher in calves as compared to cows and lesser in black hair than in white hair.
	22	Burnett et al., 2014	ELISA	• Tail switch is the ideal site for hair collection
	292	Peric et al., 2013	RIA	• As compared to Montbeliarde and Swedish Red crossbred heifers, HF cows had higher HCCs
	17	Tallo-Parra et al., 2015	EIA	• There is a high correlation among faecal and hair cortisol. • Higher HCC in black hair compared to white.
	101	Tallo-Parra et al., 2018	EIA	• High HCC is inversely related to milk production. • Higher HCC is related to a higher somatic cell count.
	45	Ghassemi et al., 2017	ELISA	• White coat cows are more resistant to heat stress.
Holstein-Friesian calves	24	Tallo-Parra et al., 2017	EIA	• No difference in HCC between a 14-day-old sample. • The difference in HCC in samples of different body region.
Holstein-Friesian and Busha breed	61	Nedić et al., 2017	ELISA	• No difference in HCC in black and white colour • Higher HCC in Holstein than Busha cows.
Hereford cross and Black Angus calves	129	Creutzinger et al., 2017	EIA	• Lower HCC in calves castrated with meloxicam compared to castration with saline.
Angus Bulls	12	Moya et al., 2013	EIA	• Greater HCC in tail hair sample compared with head and shoulder. • HCC was lower in plucked hair and higher in the hair collected by clipping.
Angus heifers	8	Schubach et al., 2016	ELISA	• Elevated HCCs in CRH-treated animals as compared to untreated animals. • HCC can be used to assess long-term HPA axis activity.
Angus×Hereford heifers	60	Schubach et al., 2016	ELISA	• Reduction in stocking density caused a considerable increase in tail switch HCC.
Dairy Cows	254	Silva et al., 2015	ELISA	• Slight variations in cattle stocking density did not affect HCC.
	196	Taminen et al., 2021	ELISA	• High HCC is associated with competition in animals and low cortisol concentration with poor environment and hygiene.
	475	Comin et al., 2013	ELISA	• Higher HCC was found in animals suffering from metritis, laminitis, and mastitis. • Elevated HCC in cows calved 1 month before sampling than normal cows.
	118	Burnett et al., 2015	ELISA	• Higher HCC in clinically diseased animals. • Multiparous animals have higher HCC than primiparous animals.
	68	Fischer- Tenhagen et al., 2018	ELISA	• No difference in HCC of primiparous and multiparous cows. • No difference in HCC in chronic lameness and healthy cows.
	142	Braun et al., 2017	LC-MS/MS	• Chronically ill animals have higher HCC compared to acutely ill animals.
	27	Braun et al., 2017	LC-MS	• Elevated HCC during the time of calving as compared to the first month of pregnancy. • Short-term stressors can be detected in 1-month-grown hair as compared to unshorn hairs.
	18	Uetake et al., 2018	EIA	• Elevated HCC in March to June irrespective of region cold or warm.

A study conducted on dairy cattle (González-de-la-Vara et al., 2011) that were given ACTH treatment three times over a period of 2 weeks reported considerably higher HCCs as compared to saline-treated or control group. Similar results were reported in goats treated repeatedly with ACTH over various time periods that ranged from 2 weeks to >2 months (Endo et al., 2018). Tallo-Para et al., (2017), treated calves two times with ACTH on day 0 and day 7 serum cortisol was altered with the injection, but in case of 14-day old hair samples, there was no change observed in HCC suggesting HCC is not altered by short term stressful conditions. Angus heifers were subjected to Corticotropin releasing hormone (CRH) administered on twice a day over a period of 2 weeks, and CRH-treated heifers were found to have elevated HCCs in comparison to control animals. Together, the finding suggests that stimulating the HPA axis repeatedly through CRH or ACTH administration results in hair shaft accumulation of cortisol. Thus, it is implied that HCC can be a tool to assess long-term HPA axis activity. Tail switch HCC was substantially increased in the case of beef cattle when the stocking density was markedly reduced from 25000 to 14 sq.m. per heifer (Schubach et al., 2017), but HCC was not affected by slight changes in the stocking density (Silva et al., 2016). Calves undergone surgical castration also had a substantially elevated HCC as compared to calves that had undergone sham-castration (Creutzinger et al., 2017). In the study conducted on 196 dairy calves from 7 to 302 days of age hair cortisol levels were estimated and reported high cortisol concentration is associated with competition in animals and low cortisol concentration with poor environment and hygiene (Tamminen et al., 2021).

Higher cortisol concentrations were found in clinically ill cows compared to healthy ones (Comin et al., 2012; Burnett et al., 2015). In the study conducted on cattle, it was reported that chronically diseased cattle had higher HCCs vs. acutely diseased cattle (Braun et al., 2017). In their study, Burnett et al., (2015) found high HCC in clinical mastitis, metritis, retained placenta, chronic ketosis, lameness, and subclinical endometritis. On the other hand, in the study, there was no significant correlation observed between chronic lameness and HCC (Fischer-Tenhagen et al., 2018). Elevated HCC was found in pregnant cows in their third trimester of pregnancy in comparison with nonpregnant animals (Braun et al., 2017), whereas, in some studies, no significant difference was noted (Tallo-Para et al., 2018). Elevated cortisol levels were found in cows calved 1 month before sampling as compared to normal cows (Comin et al., 2013). HCC increased in the spring to the summer season in cows and was lower in late summer (Comin et al., 2011). However, animals in cold temperate areas had more elevated HCC as compared to animals in warm temperate regions from March to June; it might be possible because of acclimatization to heat. The highest HCC was found from March to June and HCC was depressed from July to September June regardless of the region (Uetake et al., 2018).

Pig

Cortisol can be detected in pig hair (Table 3), the HCC of the lumbar region is higher while the HCC of the neck region is lower (Casal et al., 2017). The growth rate of hair differs from region to region, it is lowest in the neck region. In pigs, pairwise comparisons indicated the highest HCC in piglets in comparison to pigs aged 10 and 27 weeks and sows. As compared to 10 week-old pigs, adult sows showed substantially elevated HCC. HCCs were not significantly affected by the sex of the pigs. Hair color affects HCC in pigs; black hair from the same animal has more HCC in comparison to the white hair. Hair segment also significantly affects HCC; it increases from proximal to distal segment of hair (Heimburge et al., 2020). Season also has an effect on HCC in pigs and lower levels were observed in the summer season in comparison to the winter season (Bacci et al., 2014) but in another study, there was no seasonal variation in HCC, in summers HCC was 76. 4±8. 0 pg/mg and in winters it is 67. 9±8. 0 pg/mg of hair.

In lean sows, it was found that they have high HCC in comparison to sows with normal weights (Trevisan et al., 2017). Wiechers et al., (2021) in their study conducted on sows estimated HCC in both farrowing crates and loose housing systems and found no significant difference between the systems of housing. HCC in sows may be influenced by dominant stressors like farrowing and suckling piglets, and it was found that during late pregnancy and lactation, hair cortisol levels were higher than during early mid-pregnancy (Bacci et al., 2014). No significant change was observed in HCC in pigs suffering from gastro-intestinal parasites (Trevisan et al., 2017).

Sheep and goat

Ghassemi Najad et al., (2014) in their study found that wool cortisol is more reliable to measure stress than blood cortisol, they studied the effect of water restriction during heat stress in sheep and found that HCC was higher in sheep who suffered 3h water restrictions after feeding than in sheep who suffered from 2h water restrictions. Diseases and disorders can also make alterations in the HPA axis. But in this study, it was found that infection in the right hind foot in the ovine foot rot causes a decrease in hair cortisol levels. They also concluded that wool cortisone is a

Table 3: Studies of hair cortisol in pigs

Species/ breed	Number of animals	Reference	Method	Clinical findings
Hybrid Goland sows	30	Bacci et al., 2014	RIA	• Elevated HCC was found in late pregnant and lactating animals than animals in the stage of early-mid pregnancy.
Pigs (crossbred)	20	Casal et al., 2017	EIA	• HCC of the lumbar region is higher than neck region.
Pigs	28	Trevisan et al., 2017	RIA	• Lean sows have higher HCC than normal sows. • GIT parasites do not affect HCC
German Landrace pigs Saddleback pigs Crossbreed	215	Heimbürge et al., 2020	ELISA	• HCC impacted by season of sampling, age, part of the body and hair color. • Distal hair segments have more HCC. • No difference in HCC between the sexes.
Sows	61	Wiechers et al., 2021	Chemiluminescence Detection	• No significant difference of HCC was there in sows in two different for rowing systems. • HCC can be affected by dominant stressors like farrowing.

Table 4: Studies of hair cortisol in sheep and goat

Species/ breed	Number of animals	Reference	Method	Clinical findings
Corriedale female sheep	9	Ghassemi Najad et al., 2014	EIA	• High HCC under stress conditions 3hr water restriction after feeding.
Lambs	24	Stubsjoen et al., 2015	ELISA	• Hair cortisone is a more precise biomarker of chronic stress in sheep. • The wool cortisone concentration is more compared to cortisol. • Decreased HCC in case of right hind foot infection with footrot in sheep.

more reliable biomarker in comparison to wool cortisol in measuring chronic stress (Table 4, Stubsjøen et al., 2015).

Dog

In dogs, normal hair growth is influenced by several internal and external factors like seasonal variation and region of the body (Müntener et al., 2011). The hair growth rate is different for various areas of the body also it varies individually. The hair growth rate in various areas of the body was ranked as follows: shoulder > flank > forehead. During the summer season, the hair growth rate was faster as compared to (Gunaratnam and Wilkinson, 1983) the winter season in three of the dogs. Accorsi et al., (2008) in their study conducted in 29 dogs (8 females and 21 males) and 27 cats (19 females and 8 males), it was found that hair cortisol had a positive correlation with fecal and salivary cortisol. The mean cortisol level in dog hair is 2.10 ± 0.22 pg/mg. Bennett and Hayssen, (2010) in their study conducted on 48 dogs has higher HCC compared to Acrossi et al., (2008). It was found that dogs of black color have more HCC than those of other colors and the distal segment of hair has more HCC. Hair samples were acquired from the ischiatic region of dogs, and the same region was shaved after a time period ranging 6-12 weeks for hair collection. The older hair samples had a mean HCC of 12.6 pg/mg compared to 10.9 pg/mg HCC of the newer samples. Two studies in German Shephard dogs reported different HCCs, which could be attributed to different methodology, as the recovery of cortisol was higher (3.5x) when the hair was powdered rather than chopping, as practised by Accorsi et al., (2008). Nicholson and Meredith, (2015) in their study conducted in 33 dogs showed no significant difference observed among black and other color hair cortisol values. Hair cortisol in black hair and different hair colour in different breeds of dogs, no significant difference was found among various breeds.

Dogs living singularly in the house have decreased HCC levels as compared to the dogs from households with multiple dogs (Bennet and Hayssen, 2010; Grigg et al., 2017), although they had higher HCC as compared to dogs in paired housing systems (Table 5). Dogs suffering from Cushing syndrome have higher HCC than controlled healthy animals and it is helpful for the diagnosis of adrenal insufficiency and hypercortisolism (Corradini et al., 2013; Ouschan et al., 2013). Dogs suffering from atopic dermatitis had higher cortisol values in comparison to the healthy ones (Park et al., 2016). Seasonal effect on HCC has also been observed in dogs as HCC gets elevated in winters in contrast to the summer season (Roth *et al,* 2016). Van der lan et al., (2022), conducted a study on 52 sheltered dogs increased after 6 weeks in sheltered dogs A moderate positive correlation was also observed between HCC and urinary cortisol; creatinine ratio.

Horse

In horses, hair cortisol can help in measuring the HPA axis activity can be measured. Gardela et al., (2020) reported on an experiment conducted on 13 police horses (5-13 years old Pure Spanish stallions), higher HCC was associated with the relocation of horses as compared to control animals. Higher HCC was also reported during the summer season in contrast to winter and autumn season, whereas the growth rate of hair was elevated in winters. Hair cortisol concentrations in horses during the seasons of summer (5.6 ± 0.7 pg/mg) and late spring to early summer (5.5 ± 0.6 pg/mg) were found to be significantly elevated ($p < 0.05$) in comparison with winter (3.8 ± 0.4 pg/mg) and late autumn to early winter seasons (3.0 ± 0.1 pg/mg) (Olvera-Maneu et al., 2021). A study was conducted on horses (n = 153) of diverse breeds from seven stables, which included different purpose horses such as free-roaming, riding school horses, trotters etc., and it was found that the stable that had the highest frequency of events related to positive resting and social behaviours, was also the stable with the least concentration of hair cortisol among all (Sauveroche et al., 2020). Overall, no considerable difference was observed in HCC among horses managed under different regimes, but as compared to trotters and riding school horses, the free-roamers had the least negative social behaviour (Table 6). A suggestion that chronic stress level of a horse might be related to personality could be made based on the revelation that weak to moderate correlation was observed between mane hair cortisol concentration and various personality traits such as anxiousness, excitability and dominance.

HCC is correlated with body location i.e. HCC in mane samples from broodmares is elevated as compared to tail hair. Hair segments away from the root have less HCC. HCC between 1 and 2 months of post-castration is lower in castrated animals in comparison to intact animals. In a study, mare-foal pairs (n = 107) were categorized as sick and healthy foals, while in another part of the study, groups were formed on the basis of parturition in the breeding farm or hospital. There is a correlation between

Table 5: Studies of hair cortisol in dogs

Species/ breed	Number of animals	Reference	Method	Clinical findings
Labradors Retrievers German Shepherd	96	Bennet and Hayssen, 2010	EIA	• Hair and salivary cortisol were positively correlated. • Black dogs have more HCC than nonblack dogs. • Seven of Nine dogs have elevated cortisol concentration in the distal hair segment.
German Shephard	59	Roth et al, 2016	RIA	• Elevated levels of HCC in winters compared to summers.
Dog	29	Accorsi et al., 2007	EIA	• Hair and fecal cortisol were reported to be positively correlated.
	33	Nicholson and Meredith, (2015)	ELISA	• HCC of black and non-black hair did not differ significantly. • No significant difference was found between chronically ill and healthy dogs. • Decreased HCC in the dogs left alone in a single household than living in a multi-household.
	12	Grigg et al., 2017	EIA	• Declined HCC in paired housing compared to single housed dogs.
	90	Corradini et al., 2013	RIA	• Higher HCC in hypercortisolism dogs compared to healthy dogs.
	22	Ouschan et al., 2013	EIA Liquid Chromatography	• Higher HCC in hypercortisolism dogs compared to healthy dogs.
	26	Park et al., 2016	ELISA	• Dogs having atopic dermatitis have higher cortisol compared to healthy dogs.

mare and foal HCC among sick and healthy foals, implying that maternal plasma cortisol changes influence fetal hair prepartum cortisol deposition. In the third trimester of pregnancy, potential chronic stress biomarkers seem to be elevated hair DHEA-S (Dehydroepiandrosterone-sulfate) concentrated and reduced cortisol/DHEA-S ratio, and in case of sick foals, a possible indication of resilience and allostatic load. Therefore, further scrutiny is required in the assessment of prenatal HPA axis activity in horses (Lanci et al., 2022).

Wild animals

HCC provides promising results in the evaluation of chronic stress in captive animals (Table 7). The minimum sample weight required was 5 mg for hair sample collection. For consistent results, more hair should be cut during sample collection and parts of it should be used for extraction. It was found that the time of extraction, extent of grinding, body region, and hair colour cause variations in HCC in chimpanzees. But absolute HCC was not affected when sampling was done from similar areas of the body, or factors such as drying and storage. The difference in proximal and distal hair segments represented a consistent pattern in all body parts (Yamanashi et al., 2016). In chimpanzees, HCC decrease was observed along the hair shaft (Carlitz et al., 2015), while in other studies, HCC was consistent throughout the complete hair among orangutans and grizzly bears (Carlitz et al., 2014; Macbeth et al., 2010). Increased HCC was observed in relocated New Zealand white rabbits, and also when there was a change in facility staff (Peric et al., 2017). Asiatic black bears subjected to bile farming were reported to have decreased HCC when they were relocated from bile farms to a rehab facility (Malcolm et al., 2013), since bile farming is reported to have negative physical and mental effects on bears (Maas, 2000). In brown bears, chronic stress affects HCC, as HCC is inversely related to body condition and thus it is receptive to various potential long-term stressors, such as nutritional factors that might affect body condition. Short-term stressors such as those related to animal handling, capture and restraint might also affect HCC (Cattet et al., 2014). In American black bears (32 males and 32 females) it was found that HCC levels vary with sex in females the levels were 0.6 to 10.7 pg/mg which were lower compared to males having 0.5 to 35.1 pg/mg, it may be because of nutritional needs among large body male and smaller body females (Lafferty et al., 2015). Similar findings were found in coyotes (6 males and 6 females) higher HCC was found in males than females (Schell et al., 2017). In humans, males have been reported to have elevated cortisol concentrations compared to females, which might be due to the lower activity of 11β-hydroxysteroid dehydrogenase 2, a glucocorticoid-metabolising enzyme (Raven and Taylor, 1996). In contrast, it has been reported that non-human primates (Dettmer et al., 2014; Fourie et al., 2016; Laudenslager et al., 2012), polar bears (Bechshøft et al., 2011) and brown bears (Cattet et al., 2014) have considerably higher hair cortisol concentrations in females as compared to males. In a study conducted on chipmunks higher HCC was found in the summer season and is also related to docility but not related to human frequentation (Martin and Reale, 2008) Similarly higher HCC was found in black bears during the summer season (Cattet et al., 2014). Prandi et al., (2018) performed the first survey on HCC in 35 Alpine ibex 14 females and 21 males, the HCC was not affected by gender and animal's area of origin, while the average value recorded was 22.40 ±1.44 pg/mg.

Table 6: Studies of hair cortisol in horses

Species/ breed	Number of animals	Reference	Method	Clinical findings
Horses	153	Sauveroche *et al*, 2020	RIA	• Positive social and resting behaviour is associated with lower HCC.
	38	Duran et al., 2017	ELISA	• HCC in mane samples from broodmares is elevated as compared to tail hair. • Hair segments away from the root have less HCC. • HCC between 1 and 2 months of post castration is lower in castrated animals in comparison to intact animals.
	10	Olvera-Maneu et al., 2013	ELISA	• Progressive increase in HHC from winter to summer.
Mare foal pairs	107	Lanci et al., 2022	RIA	• Decreased Cortisol/DHEA-S ratio is the potential biomarker of long-term stress in the last trimester of pregnancy.

Table 7: Studies of hair cortisol in wild animals

Species/ breed	Number of animals	Reference	Method	Clinical findings
Chimpanzees	72	Yamanashi et al., 2016	EIA	• Absolute HCC was influenced by factors such as time of extraction, homogenized hair fineness and weight of a hair sample. • HCC in distal segments is higher than in proximal segments.
	78	Carlitz et al., 2015	LIA	• HCC decrease along the hair shaft.
New Zealand white rabbits	57	Peric et al., 2018	RIA	• Relocation of New Zealand white rabbits induced an increase in HCC
Bears	270	Malcom et al., 2013	EIA	• Relocation of bears to a rehabilitation facility declined HCC
	486	Cattet et al., 2014	EIA	• HCC is inversely related to the brown bear's body condition. • Females have significantly higher HCC than males.
Orangutans	71	Carlitz et al., 2014	Chemiluminescence Detection	• HCC is stable along the hair shaft.
American Black bears	116	Lafferty et al., 2015	EIA LC/MS	• HCC in males is elevated in comparison to females.
Polar bears	6 males 2 females	Bechshøft et al., 2011	EIA	• Females had elevated HCC compared to males.
Coyote	6 males 6 females	Schell et al., 2017	EIA	• More HCC in males compared to females.
Rhesus monkey	152	Dettmer et al., 2014	EIA	• Females had elevated HCC compared to males. • Increased population density is positively correlated with HCC
Non-human primates	59	Fourie et al., 2016	ELISA	• Females had elevated HCC compared to males. • Age has an affect on HCC.
	128 males 222 females	Laudenslager et al., 2012	Hair cortisol assays	• Females have higher HCC than males.

Effect of HCC on male reproduction

In order to understand how stress influences certain endocrine responses, study of the HPA axis is important (Fig. 1). Prolonged stress triggers will disrupt homeostasis and various adjustments are made by animals to cope with the stress which includes various pathological effects on metabolism, and cellular functions. Stress has been a very important risk factor affecting the fertility of animals. The neuroendocrine mechanism related to stress and its effect on male testicular function is studied. The hypothalamus PVN that regulates stress response activates the sympathetic–adrenal system (SAS), and the HPG and HPA axis pathway. Through stressor-induced stimulation of the paraventricular nucleus (PVN) in the hypothalamus and corticotrophin-releasing hormone (CRH) secretion, activation of the HPA axis takes place. Neuropeptides facilitate ACTH release through corticotroph stimulation, as ACTH acts on adrenal cortex facilitating glucocorticoids release, triggering apoptosis of Leydig cells and reduced levels of testosterone, resulting in a "knock-on" effect on the blood–testis barrier and Sertoli cells, thereby reducing spermatogenesis (Nargund et al., 2015). Peric et al., (2012) reported in breeding bulls HCC ranging from 4.5 to 18.6 pg/mg of hair, low spermatozoa concentrations were found in bulls having HCC > 10.0 pg/mg than bulls having HCC < 5pg/mg of hair. From these results, we can say that HCC can be used as a useful tool to evaluate the activity of the HPA axis in association with commencement of semen production in bulls. On the other hand, Stradaioli et al., (2017) studied bull HCCs and created two groups of HCC > 4.17 pg/mg and < 4.17 pg/mg of hair, and it was associated with spermatozoa concentration and computer-assisted sperm analysis (CASA parameters), although a significant correlation between HCC and semen characteristics could not be established. In this condition, moderate activation of the HPA axis was observed, without influencing the HPG axis with no observed effects on semen production as Moya *et al.* (2013) reported HCC ranging from 0.5 to 5.3 pg/mg of hair in Angus cross bulls. In a study conducted on

1362 men, stress was unfavorably linked with total sperm count, motility and concentration (Nordkap et al., 2020). HCC is not associated with testicular function studies conducted on 696 men. Therefore there might be a possibility that the negative effects of stress is not directly influenced by cortisol (Nordkap et al., 2022).

Effect of HCC on female reproduction

There are a variety of stressors that include environmental extremes, trauma, inflammatory reactions, and metabolic disorders possibly affecting reproduction in females through the interference of the functions of anterior pituitary, brain and ovary. These stressors result in drastic outcomes inhibition of ovulation, sexual performance, and production of livestock are also affected. Stress induces an increase in glucocorticoids which is associated with reproductive dysfunctions. Understanding how glucocorticoids might impact reproduction beyond stressful events is a necessity, even though stress is prevalent in the animals under farming system, zoos or even wildlife. Cortisol subdues tonic LH secretions through the decreasing sensitivity of the pituitary to gonadotrophin-releasing hormone (GnRH) and suppressed GnRH secretion from the hypothalamus. For cortisol to act at the hypothalamic level, gonadal steroids are necessary, although the exact mode of action is not known. Although cortisol might be sufficient to subdue tonic LH secretion, it might not be necessary in certain stressful events to suppress tonic LH secretion. In cows and sheep, there have been studies on a variety of stressors like transportation (Dobson 1987, Dobson et al., 1999, Nanda et al., 1990) which suppresses LH surge. Stress associated with inflammation/immune system depresses pulsatile LH secretion during the preovulation period of sheep (Battaglia et al., 2000). It also contributes to the delay or blockage of the LH surge in the follicular phase of certain animals like monkeys, cows and ewes (Peter et al., 1989, Xiao et al., 1998, Battaglia et al., 2000). There is a suggested mechanism in which stress influences LH and GnRH pulsatile secretion in females. It is hypothesized that cortisol increases dynorphin and/or decreases neurokinin B and kisspeptin through action on type II glucocorticoid receptors located in the kisspeptin, neurokinin

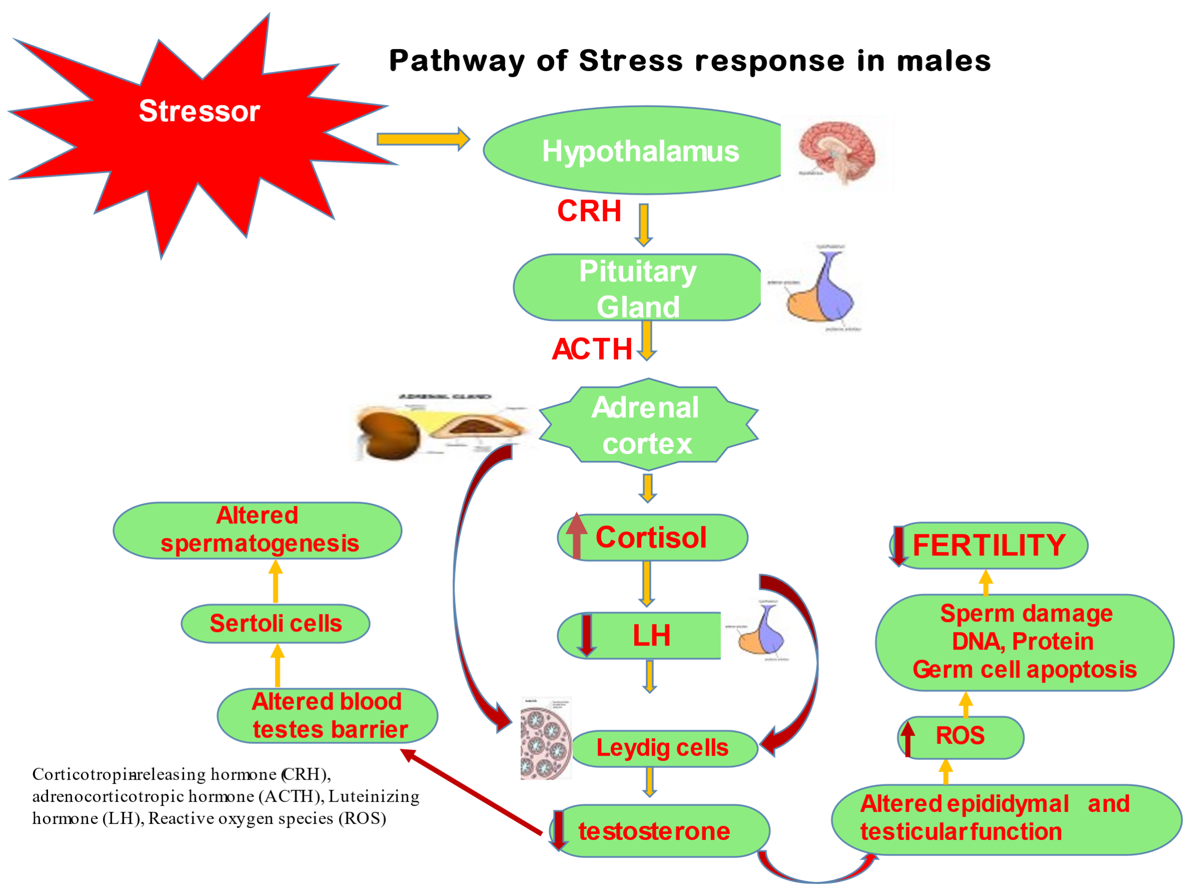

Fig. 1. The neuroendocrine mechanism related to stress and its effect on male testicular function. ACTH acts on the adrenal cortex facilitating glucocorticoids release, triggering apoptosis of Leydig cells and reduced levels of testosterone, resulting in a "knock-on" effect on the blood-testis barrier and Sertoli cells, thereby reducing spermatogenesis

B, dynorphin cells (KNDy) of the arcuate nucleus in the brain. The aforementioned changes cause GnRH neurons inhibition, alongwith hampered GnRH pulsatile secretion and the GnRH surge, thus there is inhibition of sexual behaviour and ovulation (Ralph et al., 2016).

Conclusion and future direction

Estimation of cortisol deposited in hair is gaining popularity these days, as it is a promising biomarker for HPA activation. Hair cortisol is being used as a chronic stress biomarker to check the well-being of human trauma victims, post-traumatic stress disorders (PTSD), wild animals, and lab animals. Analysis of HCC provides many benefits like noninvasive and long-term stress measured in animals. Stress can lead to delay or blockage of the LH surge in the follicular phase of certain animals like monkeys, cows, and ewes. In males, semen production is also hampered because of stress. There is damage to sperm DNA, testicular and epididymal functions are altered, the blood testes barrier is also affected. Thus, both male and female fertility is decreased. More study is needed to know the direct effect of stress on reproduction in association with a hair cortisol analysis which can be an innovative tool to evaluate fertility both in males and females. Therefore, HCC can be used as an important indicator in animal welfare research.

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Conflict of interest

We have no conflict of interest.

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