BMC Veterinary Research

SYSTEMATIC REVIEW Open Access

Analysis of different biological matrices for glucocorticoid detection in wild *Cervidae* and *Bovidae* from Europe and North America: a review

Valentina Barukčić[1*], Fiammetta Berlinguer[2], Valeria Pasciu[2], Francesca Daniela Sotgiu[2] and Nikica Šprem[1]

Abstract

Background Stressful situations that trigger an acute stress response help animals to survive in the wild. In contrast, a prolonged stressful situation can have a negative effect on the animal's health. The organism activates the HPA axis, which stimulates the adrenal cortex through an intricate network of responses. In response to the stimulation, the adrenal cortex releases glucocorticoids. Hormones and their metabolites are a good indicator of stress levels in wild animals and can be measured in many matrices such as blood, feces, urine, saliva and hair. Many studies have investigated the effects of various stressors such as anthropogenic influences, environmental and biological factors and predation on glucocorticoid levels in these non-invasive matrices. We provide an overview of the literature on this topic in wild *Cervidae* and *Bovidae*, focusing only on Europe and North America.

Results We reviewed the scientific literature published between 1979 and 2024 and found 77 papers studying the correlations between different stressors and glucocorticoid levels in wild ungulates. Most researchers used feces as the matrix of choice for analyzing glucocorticoid levels as well as enzymatic immunoassay (EIA) as the analytical method. In 41 of the 77 studies, the researchers validated the analytical method themselves (19 studies) or used the analytical method that had been previously validated by others on the studied species (22 studies).

Conclusions The increasing number of studies looking at stressful events in wild ungulates shows that researchers are interested in wildlife welfare and are making more effort to understand the biology of stress in wildlife.

Keywords Cortisol, Endocrinology, Hypothalamic-pituitary-adrenal, Stress, Ungulates

*Correspondence:
Valentina Barukčić
vbarukcic@agr.hr
[1]Faculty of Agriculture, Department of Fisheries, Apiculture, Wildlife Management and Special Zoology, University of Zagreb, Svetošimunska cesta 25, Zagreb 10000, Croatia
[2]Department of Veterinary Medicine, University of Sassari, Via Vienna 2, Sassari 07100, Italy

Background

In 1936, the young physician Hans Selye conducted an experiment with rats, exposing them to various factors such as changes in ambient temperature, loud noises, pathogens and poisons. The animals showed consistent physiological changes such as atrophy of the immune system, enlargement of the adrenal glands and peptic ulcers. Hans concluded that the rats were under stress [1]. Every organism is exposed to daily environmental changes. In addition to environmental changes, wild animals are also exposed to predation, disease and, more recently, human disturbance [2]. A natural stimulus or stressor, a predator or a storm, triggers the acute stress response system, which consists of a series of behavioural and physiological changes involving joint activity of the endocrine and nervous systems that help animals survive in the wild [3–5]. When repeated and continuous exposure causes the stressor to become chronic, the allostasis system may overstimulate the response to the persistent threat, leading to an increased risk of stress-related disease [6, 7].

As mentioned above, the immune system's response to stress is a well-organized system of intercommunication between a variety of cell types [8]. When animals find themselves in a stressful situation, the hypothalamic-pituitary-adrenal (HPA) axis is activated. Corticotrophin-releasing hormone (CRH) is released by the hypothalamus and stimulates the pituitary gland, which in turn releases the hormone adrenocorticotropin (ACTH). ACTH acts on the adrenal cortex stimulating the secretion of glucocorticoid hormones within minutes of the stressor acting on the organism [9]. Which glucocorticoid is produced depends mainly on the respective animal species. This should be taken into account when selecting a suitable assay [10]. The most important glucocorticoid hormone in most mammalian species is cortisol [9]. Cortisol plays a major role in the mobilization of energy during acute stress events and has a positive impact on the immune system [11]. Apart from the immune system, cortisol is involved in many other actions that keep the organism in homeostasis, such as the metabolism of proteins and carbohydrates, the regulation of blood pressure and anti-inflammatory actions [12]. Nevertheless, animals that are exposed to chronic stress often have a weakened immune system, lower physical fitness and their ability to reproduce is reduced. This is a result of cortisol mobilizing energy away from the systems responsible for these functions [11]. As a result of prolonged cortisol production, oxidative stress can lead to cell damage, DNA damage and damage to lipids in cell membranes, potentially affecting animal fitness and population demographics [5, 13].

Hormone assays are an important tool for understanding the basic physiological functions of an organism, such as metabolic activity, health and well-being, and reproduction. It is possible to measure hormone levels in various biological matrices, which can be divided into invasive and non-invasive, depending on the type of sampling, with blood belonging to the invasive matrices and feces, hair, saliva and urine to the non-invasive ones [14, 15]. Glucocorticoid hormones are used as indicators of stress levels in a variety of animal species. Glucocorticoid levels, including the levels of their metabolites, can also be measured in various body fluids and excretions (e.g. blood, feces, urine and saliva) as well as in hair [16].

Blood is commonly used to determine the concentration of glucocorticoids in mammals, because of its informative value, as the concentration of hormones in blood is higher than in other matrices and because of the possibility of using multiple plasma samples to assess the reaction time, frequency and amplitude of the stress response of the HPA axis. Additionally, glucocorticoids can be determined directly without the extraction and recovery process, making blood testing faster and more immediate [14, 17, 18]. It must be drawn via venipuncture, which makes this method invasive. Capturing, handling, restraining the animals and venipuncture are stressful procedures, especially in wild animals, which can activate the HPA axis and lead to possible differences in glucocorticoid concentrations [14, 17, 19]. In addition to sampling from live animals, blood samples can also be taken from culled animals, but in this case it is difficult to compare the glucocorticoid concentrations of live and dead animals [20, 21]. The type of trauma suffered by the dead animals has a strong influence on blood glucocorticoid levels and should be taken into account when interpreting the results. Animals culled immediately have lower cortisol levels than animals culled after trauma [22]. Blood is used for monitoring acute stress levels that are influenced by short-term hormonal changes [23]. Repeated collection of blood samples from non-domesticated and wild animals is very difficult, and in many cases even impossible [21]. When taking blood samples, researchers must consider several things, such as the welfare of the animals, the need for anesthesia, and the safety of the researcher. These are all reasons why non-invasive matrices are gaining popularity in the monitoring of stress in wildlife [24].

The hormone cortisol can be found in the blood in two ways: bound to proteins and unbound or free cortisol. Around 90–95% of the total amount of the hormone is bound to corticosteroid-binding globulin (CBG), a very large protein that is mainly produced by the liver. Unbound cortisol can easily penetrate membranes so that it is available for diffusion into tissues and has the biological activity required to regulate metabolic and immunological processes, whereas bound cortisol is too large to penetrate cell membranes, and therefore cannot reach intracellular receptors [19, 25]. The unbound

cortisol is absorbed into the tissues (hair) and extracellular fluids such as saliva and is available for metabolism and processing by the liver whereupon it is excreted into the intestine via bile, with the glucocorticoid metabolites becoming measurable in the feces and urine, reflecting the biologically active portion of the hormones [25, 26].

The measurement of glucocorticoid metabolites in the feces of wild and domestic animals is increasingly used in endocrinology studies, as it offers many advantages during sampling [24, 27]. Samples can be easily obtained in the field without disturbing and capturing the animals, thus avoiding the risk of harming them. Since fecal samples are one of the non-invasive sampling methods, analysis of the samples can provide an accurate assessment of stress levels without the acute increase in glucocorticoid levels caused by handling the animals. In addition, the ability to repeat sampling at specific intervals allows for more detailed and accurate results than invasive methods [28]. The glucocorticoid metabolites in feces represent an average level of circulating hormones over a longer period of time rather than just a point sample and thus provide a more accurate measure of chronic stress levels [29]. A potential disadvantage is that the fecal samples should be collected fresh. Donini et al. [30] conducted a study in which they examined the temporal stability of fecal glucocorticoid metabolites in red deer *Cervus elaphus* and northern chamois *Rupicapra rupicapra* over a 7-day period. Fecal samples were exposed to natural weather conditions on top of Monte Sole. The results showed that glucocorticoid levels rapidly decreased from the first day, up to the 4th day of the experiment and remained stable thereafter. The glucocorticoid metabolite levels degrade in the feces, which may be crucial when drawing conclusions.

Urine can be used as a matrix for analyzing the content of glucocorticoid metabolites, as the kidneys filter the glucocorticoids metabolized by the liver. Urine collection is also considered a non-invasive method with minimal human interaction with the animals and it is less under the influence of short-term stressors, suggesting that it can be used to monitor chronic stress levels [19, 31]. Urine as a matrix is less commonly used by researchers because it is difficult to collect in the field [23, 24]. However, according to Palme et al. [26], it is an excellent tool for monitoring glucocorticoids as a large proportion is excreted via urine. White et al. [31] collected urine from wild elks *Cervus canadensis* from the snow. Danish et al. [32] tested three urine collection devices. The best performing device was the Salivette synthetic device, which is best suited for collecting urine from the ground.

Sampling saliva is considered less invasive than the collection of blood samples because it requires less human handling [33]. The correlation between salivary and blood glucocorticoid levels is considered high as it reflects the concentration of glucocorticoids in the blood [24, 34]. According to Sheriff et al. [24], the method should first be validated for use in each species, as the glucocorticoids in saliva vary between 10 and 60% of those in blood plasma, depending on the species in question. The use of saliva as a matrix in wild animals can be difficult as there may be hurdles that limit researchers in saliva collection, whereas captive and domestic animals can be successfully trained for saliva collection [35].

Recently, hair has become increasingly popular as a matrix for the study of stress hormones in wild animals. Free cortisol is indeed incorporated in hair follicles via the bloodstream [36]. Hair can be easily collected using hair traps, which are considered minimally invasive techniques [37]. No special method is required to store the samples as glucocorticoid concentrations are stable for months and even years, making them a very convenient matrix. During hair growth, hormone levels accumulate in the hair and are therefore considered an indicator of long-term physiological processes [23, 38]. One disadvantage is the need to determine hair growth to determine the time period during which glucocorticoid metabolites have accumulated [23]. As previously mentioned, hair can be easily collected from living animals, but it can also be collected from dead animals and used for analysis [39].

In endocrinology, immunoassays such as the Enzyme immunoassay (EIA) (of which one specific application is the enzyme-linked immunosorbent assay (ELISA)) and the radioimmunoassay (RIA) are still the methods of choice for many researchers due to their high sensitivity. Both assays can be used for the determination of glucocorticoid levels in different matrices [40]. The main differences between EIA and RIA are that EIA assays are safer, faster, more economical and more environmentally friendly than RIA assays [41, 42]. On the other hand, the RIA assay is more sensitive and generally has a lower detection limit than the EIA [43]. When using both methods to analyze hormone levels, it is important to perform analytical and biological validation of the method used for the matrix in question. Validation of the method is of great importance as it confirms the reliability of the results [44, 45].

In this review, we discuss the variety of matrices in which glucocorticoids, particularly cortisol, can be measured and the different environmental and non-environmental influences on their concentrations. In particular, we aim to *i*) provide an overview of all matrices in which cortisol has been measured in wild *Cervidae and Bovidae* to date, *ii*) provide an overview of all influences on cortisol levels that have been studied in wild *Cervidae and Bovidae* to date, *iii*) review which analytical methods (EIA or RIA) have been used to determine cortisol levels in different matrices and whether or not they have been

validated, and *iv*) perform a trend analysis of publications to gain insight into the increase or decrease in publication numbers over time.

Methods

In order to find the relevant literature, a systematic search was carried out in the data sources Web of Science, Google Scholar and Scopus, whereby the period of publication of the articles was not specified. The following keywords were used in the literature search: 'ungulates', 'wild ungulates', '*Cervidae*', '*Bovidae*' 'stress', 'cortisol', 'cortisol levels', 'glucocorticoids'. The databases provided 124 studies on glucocorticoid levels, but many of the studies did not meet our requirements and were excluded from the search [Additional file 1]. Only peer-reviewed scientific papers in which the species studied were located in Europe and North America were considered, as *Cervidae* and *Bovidae* from these two continents have significantly similar ecological and biological characteristics. All papers were written in English. In the literature search, we found 77 peer review papers from 1979 to 2024 that provided data on various impacts on cortisol levels in different matrices from different taxa [see Additional file 2]. Depending on the matrix used to determine hormone levels in different taxa, the studies examined were divided into five categories: blood, feces, urine, saliva and hair. The papers found in the database search were also categorized according to the validation of the methods used (RIA or EIA) for the quantification of glucocorticoid levels. If no method validation information was provided in the paper, the method used was considered unvalidated. The information on the stressors that had an impact on glucocorticoid levels and their metabolites was categorized into four groups according to the type of impact: anthropological, environmental, biological and uncategorized.

The publication years of all scientific papers were used for trend analysis. For each year, the number of published papers was appended, and the level 3 polynomial autoregressive Poisson model was used to create a visual representation of the publication trend over the years.

Results

Out of the total 77 peer review scientific papers examined, 51 relate to the European continent and 26 to the North American continent. The study areas included 14 countries on the European continent and 16 states on the North American continent. We have recorded the number of published scientific papers per country/state and used this information to create a map representing the number of published scientific papers by geographical distribution (Fig. 1).

These studies were performed in a total of 17 taxa [see Additional file 2] from the groups of *Cervidae* and *Bovidae* (Fig. 2). Of the species belonging to the *Cervidae* family, we calculated the percentage of each species in the same family whose cortisol levels were analyzed. The species belonging to the *Cervidae* family are red deer (30.2%), roe deer *Capreolus capreolus* (15.9%), elk (9.5%), mule deer *Odocoileus hemionus* (3.2%), fallow deer *Dama dama* (6.3%), axis deer *Axis axis* (1.6%), white-tailed deer *Odocoileus virginianus* (11.1%), black-tailed deer *O. hemionus columbianus* (3.2%), moose *Alces alces* (7.9%) and finally reindeer *Rangifer tarandus* (11.1%). The percentages of studies carried out on ungulate species from the *Bovidae* family were also calculated for each species, more specifically for the chamois (55%), the bighorn sheep *Ovis canadensis* (5%), the Alpine ibex *Capra ibex* (10%), the Iberian ibex *Capra pyrenaica* (5%), the mountain goats *Oreamnos americanus* (10%), the American bison *Bison bison* (10%) and the European bison *Bison bonasus* (5%). Nineteen studies were conducted on the red deer, making it the most studied species of all 17 taxa considered in this review. When searching the databases, we found only one study for each of the following species: Axis deer, Iberian ibex and European bison [46–48].

On the European continent, there are eleven species belonging to the *Cervidae* family, and glucocorticoid levels have been studied in five of them. In addition, there are nine species from the *Bovidae* family on the European continent, of which only four have been tested for glucocorticoid levels. On the North American continent, out of eight *Cervidae* and seven *Bovidae* species, respectively six and three of them were used to assess the concentrations of glucocorticoids and their metabolites.

Matrices results

Of the 77 papers we found in the database search, 16 used only blood as the matrix for glucocorticoid analysis and three used blood and other matrices to determine glucocorticoid levels in various ungulate species, in particular red deer, roe deer, fallow deer, axis deer, reindeer, white-tailed deer, moose and Alpine ibex. In one study, the cortisol levels in the blood of five ungulate species were compared, with the cortisol levels of roe deer differing significantly from each other and from the group of red deer, fallow deer and moose. The glucocorticoid levels of red deer, fallow deer and moose did not differ significantly from each other [22]. Of the 16 studies mentioned above, 11 studied free ranging wild animals and five studies were conducted on captive and farmed animals. In addition, blood samples from live animals were used in 10 of the included studies and blood samples from hunted animals were used in six of the studies. The blood samples were centrifuged and frozen until further analysis. The percentage of studies in which the time of blood sampling was reported was 79%. In the remaining

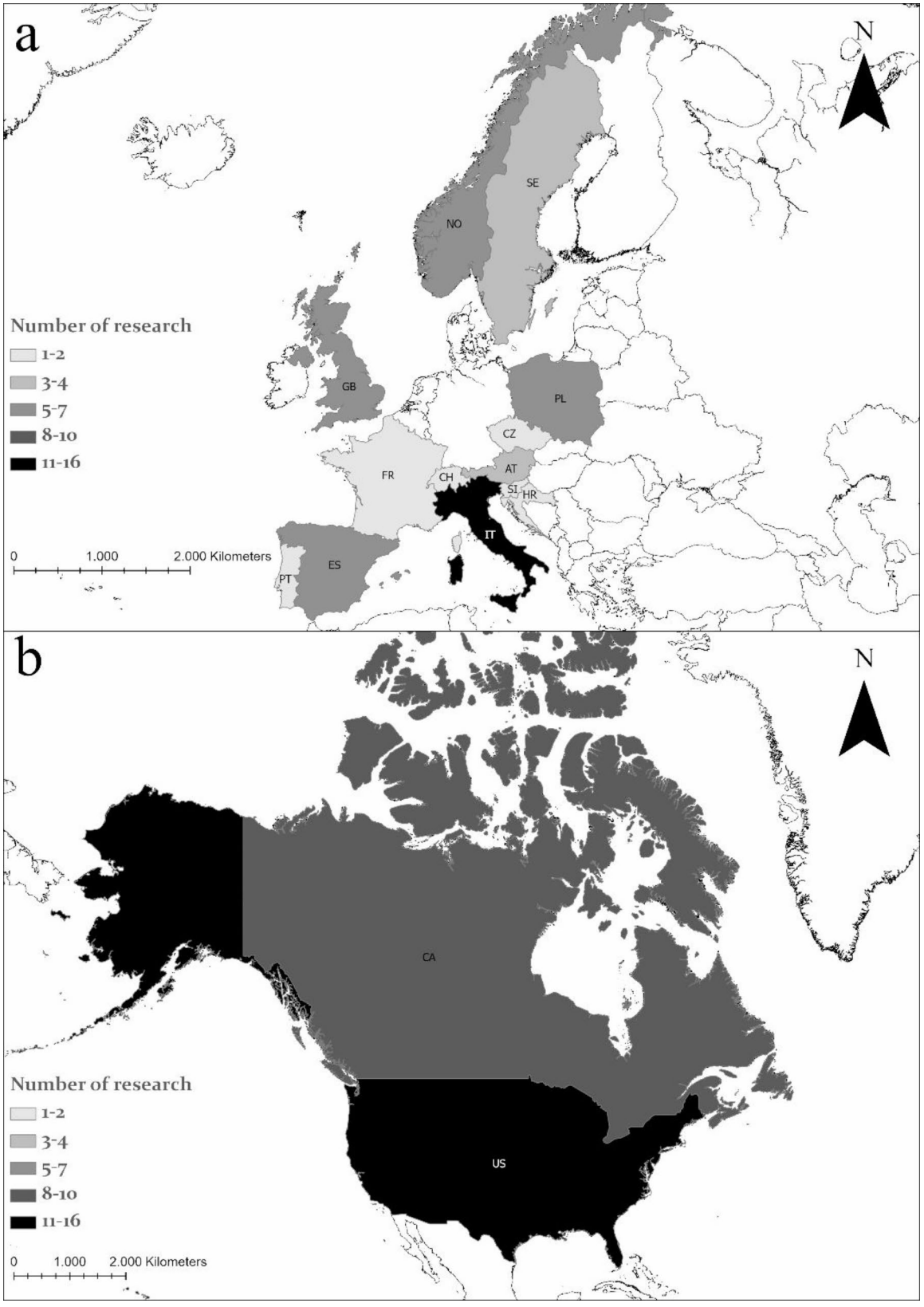

Fig. 1 Geographic representation of published scientific paper per country/state on the stressful impacts on cortisol levels: (**a**) Europe and (**b**) North America

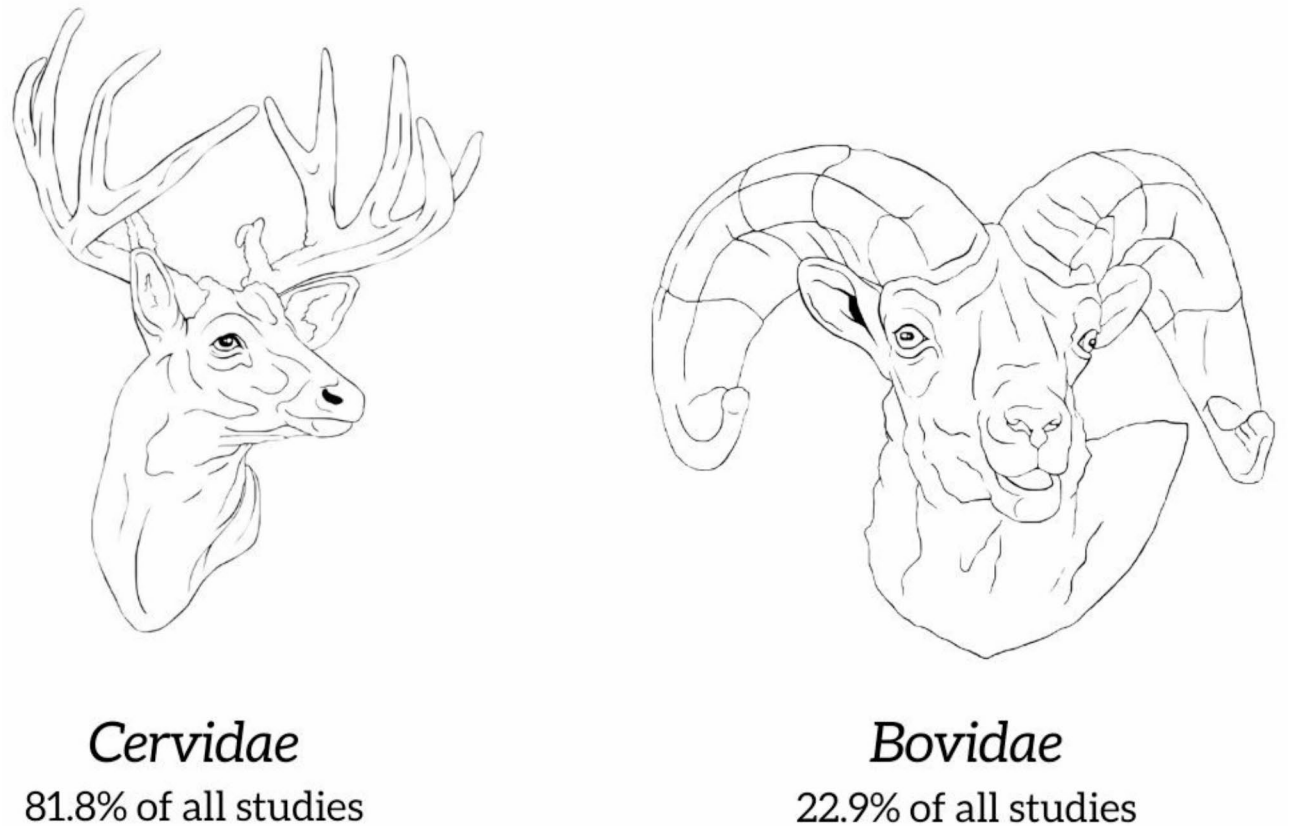

Fig. 2 Representation of studies that have investigated species of wild ungulates divided into two families. The total is 104.7% as some of the studies used matrices to detect glucocorticoid levels in species from both families

21% of the studies, the time of sampling was not specified. The time of sample collection is described in Table 1.

To measure glucocorticoid levels in the blood, in 16 studies the blood samples were only centrifuged, and the serum or plasma was used to quantify the hormone concentration indicating that the total level of the glucocorticoid was measured. Blood extraction was performed in three studies. In one study, 10-fold volume of tert-butyl methyl ether was used for extraction. The samples were then frozen at − 60 °C, the extracts were decanted, evaporated and reconstituted [49]. In another study, five mL of diethyl ether was added to the centrifuged plasma and the samples were shaken and centrifuged again, after which they were frozen. After freezing, the remaining liquid component was separated and dried at 40 °C under a stream of nitrogen. The procedure was carried out twice to increase the yield of the extraction process [50]. In the third study, 0.2 mL was mixed with five mL of petroleum ether or diethyl ether. After centrifugation, the supernatant was completely evaporated under a suction hood with air flow at 37 °C [51]. These extraction procedures performed in the three studies indicate that free glucocorticoid levels in the blood were quantified. However, none of the studies specified if the target of their analysis was free, bound or total glucocorticoid concentration.

Forty-one of the 77 studies used feces as the matrix for examining cortisol levels, and six studies included feces and other matrices in their research. Fecal samples were taken from red deer, roe deer, fallow deer, white-tailed deer, black-tailed deer, mule deer, elk, moose, and reindeer from the cervid family and from chamois, Alpine ibex, Iberian ibex, bighorn sheep, mountain goats, and American and European bison from the bovid family. In almost all studies, fresh fecal samples were collected for glucocorticoid metabolite analysis, with the exception of one study in which the condition of the fecal samples was not specified. The exact time of collection of the samples after defecation was reported in only 36% of the studies. The other 64% of the studies did not specify the time after defecation, but determined the freshness of the samples based on their condition (Table 1).

Of the studies that used feces as a matrix, six studies used homogenized subsamples for glucocorticoid quantification, 16 studies only mentioned that homogenized samples were used for further analysis, while three studies mentioned that samples were divided into subsamples without homogenization procedures. The rest of the studies (i.e. 22) did not specify whether the samples were divided into subsamples or homogenized.

Table 1 Variables affecting glucocorticoids concentrations in all matrices and timing of sample collection

Matrices	Species	Variables	Time of sample collection	References
Blood, feces, urine, saliva, hair	Alpine Ibex - *Capra ibex*	Season, sex, age	Feces immediately after defecating, all other matrices immediately after restraining	Kastelic M, Gregurić Gračner G, Tomažič I, Kvapil P, Harej M, Dovč A. Comparison of Cortisol Concentrations inDifferent Matrices in Alpine Ibex (Capra ibex) at the Zoo. Animals (2023) 13: 2491
Blood	Reindeer - *Rangifer tarandus*	Season, sex	10, 20, 30 min after imobilisation	Ringberg T. The Spitzbergen reindeer-a winter-dormant ungulate? Acta Physiologica Scandinavica (1979) 105: 268–273
Blood	White-tailed deer - *Odocoileus virginianus*	Cold periods vs. warm periods	Every 60 min	Bubenik GA, Bubenik AB, Schams D, Leatherland JF. Circadian and circannual rhythms of LH, FSH, testosterone (T), prolactin, cortisol, T3 and T4 in plasma of mature, male white-tailed deer. Comparative Biochemistry and Physiology Part A: Physiology (1983) 76: 37–45.
Blood	White-tailed deer - *Odocoileus virginianus*	Season	15 to 20 min after imobilisation	Bubenik GA, Leatherland JF. Seasonal levels of cortisol and thyroid hormones in intact and castrated mature male white-tailed deer. Canadian Journal of Zoology (1984) 62: 783–787.
Blood	Reindeer - *Rangifer tarandus*	Season	Immediately after death	Nilssen KJ, Bye K, Sundsfjord JA, Blix AS. Seasonal changes in T3, FT4, and cortisol in free-ranging Svalbard reindeer (Rangifer tarandus platyrhynchus). General and Comparative Endocrinology (1985) 59: 210–213.
Blood	Axis deer - *Axis axis*	Season	10, 20, 30 min after imobilisation	Bubenik GA, Brown RD. Seasonal levels of cortisol triiodothyronine and thyroxine in male axis deer. Comparative Biochemistry and Physiology Part A: Physiology (1989) 92: 499–503.
Blood	White-tailed deer - *Odocoileus virginianus*	Season	30 min after imobilisation	Delgiudice GD, Mech LD, Kunkel KE, Gese EM, Seal US. Seasonal patterns of weight, hematology, and serum characteristics of free-ranging female white-tailed deer in Minnesota. Canadian Journal of Zoology (1992) 70: 974–983.
Blood	Red deer - *Cervus elaphus*	Hunting	As soon as possible after death, no specific time	Bateson P, Bradshaw EL. Physiological effects of hunting red deer (Cervus elaphus). Proceedings of the Royal Society of London. Series B: Biological Sciences (1997) 264: 1707–1714
Blood	Reindeer - *Rangifer tarandus*	Season	10, 20, 30 min after imobilisation	Bubenik GA, Schams D, White RG, Rowell J, Blake J, Bartos L. Seasonal levels of metabolic hormones and substrates in male and female reindeer (Rangifer tarandus). Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology (1998)120: 307–315.
Blood	Red deer - *Cervus elaphus*	Wounding, huntig	After death, no specific time	Bateson P, Bradshaw EL. The effects of wound site and blood collection method on biochemical measures obtained from wild, free-ranging red deer (Cervus elaphus) shot by rifle. Journal of Zoology (2000) 252: 285–292.
Blood	Red deer - *Cervus elaphus*	Social rank, testosterone	Immediately after death	Bartoš L, Schams D, Bubenik GA, Kotrba R, Tománek M. Relationship between rank and plasma testosterone and cortisol in red deer males (Cervus elaphus). Physiology & Behavior (2010) 101: 628–634.
Blood	Reindeer - *Rangifer tarandus*	Human handling, induction time	30 min from imobilisation	Miller AL, Evans AL, Os Ø, Arnemo JM. Biochemical and hematologic reference values for free-ranging, chemically immobilized wild Norwegian reindeer (Rangifer tarandus tarandus) during early winter. Journal of Wildlife Diseases (2013) 49: 221–228.
Blood	Roe deer - *Capreolus capreolus*	Predation, humans, season, age	After capturing, no specific time	Bonnot NC, Bergvall UA, Jarnemo A, Kjellander P. Who's afraid of the big bad wolf? Variation in the stress response among personalities and populations in a large wild herbivore. Oecologia (2018) 188: 85–95.
Blood	Roe deer, moose, red deer, fallow deer, wild boar - *Capreolus capreolus*, *Cervus elaphus*, *Dama dama*, *Sus scrofa*	Hunting method, effect of trauma	As soon as possible, max 1 to 2 h after death	Gentsch RP, Kjellander P, Röken BO. Cortisol response of wild ungulates to trauma situations: hunting is not necessarily the worst stressor. European Journal of Wildlife rResearch, (2018) 64: 1–12.
Blood	Reindeer - *Rangifer tarandus*	Human handling	Immediately after capture	Trondrud LM, Ugland C, Ropstad E, Loe LE, Albon S, Stien A, Evans AL, Medbøe Thorsby P, Veiberg V, Irvine RJ, Pigeon, G. Stress responses to repeated captures in a wild ungulate. Scientific Reports (2022) 12: 16,289.

Table 1 (continued)

Matrices	Species	Variables	Time of sample collection	References
Blood	Roe deer - *Capreolus capreolus*	Sex, hunting season, age	Immediately after death	Dziki-Michalska K, Tajchman K, Kowalik S. Physiological response of roe deer (Capreolus capreolus) during stalking hunts depending on age. BMC Veterinary Research (2023) 19: 266.
Blood	Red deer - *Cervus elaphus*	Hunting, sex, age	5 min after death	Dziki-Michalska K, Tajchman K, Kowalik S, Wójcik M. The Levels of Cortisol and Selected Biochemical Parameters in Red Deer Harvested during Stalking Hunts. Animals (2024) 14: 1108.
Blood, feces, hair	Red deer - *Cervus elaphus*	Hunting activities, sex, age, season	1 to 4 h after death	Vilela S, Alves da Silva A, Palme R, Ruckstuhl KE, Sousa JP, Alves J. Physiological stress reactions in red deer induced by hunting activities. Animals (2020) 10: 1003.
Blood, hair	Red deer - *Cervus elaphus*	Pregnant females	After death, no specific time	Ventrella D, Elmi A, Bertocchi M, Aniballi C, Parmeggiani A, Govoni N, Bacci ML. Progesterone and cortisol levels in blood and hair of wild pregnant red deer (Cervus elaphus) hinds. Animals (2020) 10: 143.
Feces	Elk - *Cervus canadensis*	Season, sex, human activity	No specific time, fresh samples	Millspaugh JJ, Woods RJ, Hunt KE, Raedeke KJ, Brundige GC, Washburn BE, Wasser SK. Fecal glucocorticoid assays and the physiological stress response in elk. Wildlife Society Bulletin (2001) 899–907.
Feces	Elk - *Cervus canadensis*	Snowmonile activity/ human presence	No specific time, fresh samples	Creel S, Fox JE, Hardy A, Sands J, Garrott B, Peterson RO. Snowmobile activity and glucocorticoid stress responses in wolves and elk. Conservation Biology (2002) 16: 809–814.
Feces	Red deer - *Cervus elaphus*	Sex, season	No specific time, fresh samples	Huber S, Palme R, Arnold W. Effects of season, sex, and sample collection on concentrations of fecal cortisol metabolites in red deer (Cervus elaphus). General and Comparative Endocrinology (2003) 130: 48–54.
Feces	Bighorn sheep - *Ovis canadensis*	Season, lungworms	After defecation, no specific time, fresh samples	Goldstein EJ, Millspaugh JJ, Washburn BE, Brundige GC, Raedeke KJ. Relationships among fecal lungworm loads, fecal glucocorticoid metabolites, and lamb recruitment in free-ranging Rocky Mountain bighorn sheep. Journal of Wildlife Diseases (2005) 41: 416–425.
Feces	Chamois - *Rupicapra rupicapra*	Sex, parasites, season	No specific time, fresh samples	Hoby S, Schwarzenberger F, Doherr MG, Robert N, Walzer C. Steroid hormone related male biased parasitism in chamois, Rupicapra rupicapra rupicapra. Veterinary Parasitology (2006) 138: 337–348.
Feces	American Bison - *Bison bison*	Rutting season	No specific time, fresh samples	Mooring MS, Patton ML, Lance VA, Hall BM, Schaad EW, Fetter GA, Frotin SS, McPeak KM. Glucocorticoids of bison bulls in relation to social status. Hormones and Behavior (2006) 49: 369–375.
Feces	Chamois - *Rupicapra rupicapra*	Season, fecal nitrogen, human disturbances	No specific time, fresh samples	Dalmau A, Ferret A, Chacon G, Manteca X. Seasonal changes in fecal cortisol metabolites in Pyrenean chamois. The Journal of Wildlife Management (2007) 71: 190–194.
Feces	White-tailed deer - *Odocoileus virginianus*	Winter progression, diet quality, social rank	No longer than 6 h	Taillon J, Côté SD. Are faecal hormone levels linked to winter progression, diet quality and social rank in young ungulates? An experiment with white-tailed deer (Odocoileus virginianus) fawns. Behavioral Ecology and Sociobiology (2008) 62: 1591–1600.
Feces	Elk - *Cervus canadensis*	Predation, season, sex	No specific time, fresh samples	Creel S, Winnie Jr JA, Christianson D. Glucocorticoid stress hormones and the effect of predation risk on elk reproduction. Proceedings of the National Academy of Sciences (2009) 106: 12,388–12,393.
Feces	Red deer - *Cervus elaphus*	Season, temperature, percipitation, water level	No specific time, fresh samples	Corlatti L, Palme R, Frey-Roos F, Hackländer K. Climatic cues and glucocorticoids in a free-ranging riparian population of red deer (Cervus elaphus). Folia Zoologica (2011) 60: 176–180.
Feces	Fallow deer - *Dama dama*	Season	No specific time, fresh samples	Konjević D, Janicki Z, Slavica A, Severin K, Krapinec K, Božić F, Palme R. Non-invasive monitoring of adrenocortical activity in free-ranging fallow deer (Dama dama L.). European Journal of Wildlife Research (2011) 57: 77–81.
Feces	Chamois - *Rupicapra rupicapra*	Rutting season	No specific time, fresh samples	Corlatti L, Béthaz S, von Hardenberg A, Bassano B, Palme R, Lovari S. Hormones, parasites and male mating tactics in Alpine chamois: identifying the mechanisms of life history trade-offs. Animal Behaviour (2012) 84: 1061–1070.
Feces	Chamois - *Rupicapra rupicapra*	Tourism, number of visitors, season	No specific time, fresh samples	Zwijacz-Kozica T, Selva N, Barja I, Silván G, Martínez-Fernández L, Illera JC, Jodłowski M. Concentration of fecal cortisol metabolites in chamois in relation to tourist pressure in Tatra National Park (South Poland). Acta Theriologica (2013) 58: 215–222.

Table 1 (continued)

Matrices	Species	Variables	Time of sample collection	References
Feces	Chamois - *Rupicapra rupicapra*	Age, social status, temp, snow depth, percipitation, season	Soon after defecation, no specific time	Corlatti L, Palme R, Lovari S. Physiological response to etho-ecological stressor in male Alpine chamois: timescale matters! Naturwissenschaften (2014) 101: 577–586.
Feces	Alpine Ibex - *Capra ibex*	Chemical imobilisation	No specific time	Brivio F, Grignolio S, Sica N, Cerise S, Bassano B. Assessing the impact of capture on wild animals: the case study of chemical immobilisation on alpine ibex. PLoS One (2015) 10: e0130957.
Feces	Chamois - *Rupicapra rupicapra*	Season, sex, study area	No specific time, fresh samples	Hadinger U, Haymerle A, Knauer F, Schwarzenberger F, Walzer C. Faecal cortisol metabolites to assess stress in wildlife: evaluation of a field method in free-ranging chamois. Methods in Ecology and Evolution (2015) 6: 1349–1357.
Feces	Red deer - *Cervus elaphus*	Month (Season), age, harem size,	Within 5 min of defecation	Pavitt AT, Walling CA, Möstl E, Pemberton JM, Kruuk LE. Cortisol but not testosterone is repeatable and varies with reproductive effort in wild red deer stags. General and Comparative Endocrinology (2015) 222: 62–68.
Feces	Fallow deer- *Dama dama*	Season, captive vs. free-ranged	After defecation, no specific time, fresh samples	Konjević D, Janicki Z, Slavica A, Severin K, Krapinec K, Želježić D, Božić F. Monitoring cortisol metabolites in the faeces of captive fallow deer (Dama dama L.). Veterinarski Arhiv (2016) 86: 363–371.
Feces	Black-Tailed deer - *O. heinosus columbianus*	Month (Season), two populations, food abudance	No specific time, fresh samples	Le Saout S, Massouh M, Martin JL, Presseault-Gauvin H, Poilvé E, Côté SD, Picot D, Verheyden H, Chamaillé-Jammes, S. Levels of fecal glucocorticoid metabolites do not reflect environmental contrasts across islands in black-tailed deer (Odocoileus hemionus sitkensis) populations. Mammal Research (2016) 61: 391–398.
Feces	Red deer - *Cervus elaphus*	Age, season, reproductive state	Within 5 min of witnessing defecation	Pavitt AT, Pemberton JM, Kruuk LE, Walling CA. Testosterone and cortisol concentrations vary with reproductive status in wild female red deer. Ecology and Evolution, (2016) 6: 1163–1172.
Feces	Fallow deer - *Dama dama*	Hunting	After defecation, no specific time, fresh samples	Pecorella I, Ferretti F, Sforzi A, Macchi E. Effects of culling on vigilance behaviour and endogenous stress response of female fallow deer. Wildlife Research (2016) 43: 189–196.
Feces	Chamois - *Rupicapra rupicapra*	Food availability, group size,	Immediately after defecation was observed	Fattorini N, Brunetti C, Baruzzi C, Macchi E, Pagliarella MC, Pallari N, Lovari S, Ferretti F. Being "hangry": Food depletion and its cascading effects on social behaviour. Biological Journal of the Linnean Society (2018) 125: 640–656.
Feces	Chamois - *Rupicapra rupicapra*	Age, season.	Immediately after defecation was observed	Fattorini N, Lovari S, Brunetti C, Baruzzi C, Cotza A, Macchi E, Pagliarella MC, Ferretti F. Age, seasonality, and correlates of aggression in female Apennine chamois. Behavioral Ecology and Sociobiology (2018) 72: 1–17.
Feces	Chamois - *Rupicapra rupicapra*	Domestic animals, reed deer, hikers	After defecation, no specific time, fresh samples	Formenti N, Viganó R, Fraquelli C, Trogu T, Bonfanti M, Lanfranchi P, Palme R, Ferrari N. Increased hormonal stress response of Apennine chamois induced by interspecific interactions and anthropogenic disturbance. European Journal of Wildlife Research (2018) 64: 1–8.
Feces	Roe deer - *Capreolus capreolus*	Urbanisation- road type and noise values, distance from the road	No specific time, fresh samples	Iglesias-Merchan C, Horcajada-Sánchez F, Diaz-Balteiro L, Escribano-Ávila G, Lara-Romero C, Virgós E, Planillo A, Barja I. A new large-scale index (AcED) for assessing traffic noise disturbance on wildlife: stress response in a roe deer (Capreolus capreolus) population. Environmental Monitoring and Assessment (2018) 190: 1–16.
Feces	Red deer - *Cervus elaphus*	Enviromental factors, human factors, sex, age, season	Directly from rectum	Santos JP, Acevedo P, Carvalho J, Queiros J, Villamuelas M, Fonseca C, Gortázar C, López-Olvera JR, Vicente J. The importance of intrinsic traits, environment and human activities in modulating stress levels in a wild ungulate. Ecological Indicators (2018) 89: 706–715.
Feces	Red Deer, Roe deer - *Cervus elaphus*, *Capreolus capreolus*	Natural and anthropogenic factors, predation	No specific time, fresh samples	Zbyryt A, Bubnicki JW, Kuijper DP, Dehnhard M, Churski M, Schmidt K. Do wild ungulates experience higher stress with humans than with large carnivores? Behavioral Ecology (2018) 29: 19–30.

Table 1 (continued)

Matrices	Species	Variables	Time of sample collection	References
Feces	Roe deer - *Capreolus capreolus*	Habitat, livestock presence	No specific time, fresh samples	Horcajada-Sánchez F, Escribano-Ávila G, Lara-Romero C, Virgós E, Barja I. The effect of livestock on the physiological condition of roe deer (Capreolus capreolus) is modulated by habitat quality. Scientific Reports (2019) 9: 15,953.
Feces	Iberian ibex - *Capra pyrenaica*	Infestation of Sarcoptes scabiei	Directly from rectum or immediately after deposition	Pérez JM, Molina L, Ureña-Gutiérrez B, Espinosa J, López-Montoya AJ, Boos M, Granados JE, Cano-Manuel FJ, Azorit C. Individual stress responses to Sarcoptes scabiei infestation in Iberian ibex, Capra pyrenaica. General and Comparative Endocrinology (2019) 281: 1–6.
Feces	Mule deer - *Odocoileus hemionus*	Competition with elk, harsh and mild winter	Fresh samples, up to to 2 h after defecation	Atwood MP, Kie JG, Millspaugh JJ, Matocq MD, Bowyer RT. Condition of mule deer during winter: stress and spatial overlap with North American elk. Mammal Research (2019) 65:349–58.
Feces	Roe deer - *Capreolus capreolus*	Urbanisation, temperature, day-night	Directly from rectum	Carbillet J, Rey B, Palme R, Morellet N, Bonnot N, Chaval Y, Cargnelutti B, HewisonAJM, Gilot-Fromont E, Verheyden H. Under cover of the night: Context-dependency of anthropogenic disturbance on stress levels of wild roe deer Capreolus capreolus. Conservation Physiology (2020) 8: coaa086.
Feces	Elk - *Cervus canadensis*	Hunting season, group size, food availability,	Directly from rectum	Ensminger DC, Pritchard C, Langkilde T, Gingery T, Banfield JE, Walter WD. The influence of hunting pressure and ecological factors on fecal glucocorticoid metabolites in wild elk. Wildlife Biology (2020) 2020: 1–7.
Feces	Roe deer - *Capreolus capreolus*	Wind farms, predation	No specific time, fresh samples	Klich D, Łopucki R, Ścibior A, Gołębiowska D, Wojciechowska M. Roe deer stress response to a wind farms: Methodological and practical implications. Ecological Indicators (2020) 117: 106,658.
Feces	Red deer, chamois - *Cervus elaphus*, *Rupicapra rupicapra*	Season, snow height, forage quality, human disturbence	No specific time, fresh samples	Anderwald P, Andri SC, Palme R. Reflections of ecological differences? Stress responses of sympatric Alpine chamois and red deer to weather, forage quality, and human disturbance. Ecology and Evolution (2021) 11:15740–15,753.
Feces	Red deer - *Cervus elaphus*	Tourism	After defecation, no specific time, fresh samples	Dixon G, Marriott AS, Stelfox G, Dunkerley C, Batke SP. How do red deer react to increased visitor numbers? A case study on human-deer encounter probability and its effect on cortisol stress responses. Nature Conservation (2021) 43: 55–78.
Feces	Red deer - *Cervus elaphus*	Testosteron, size of antlers	Directly from rectum	de La Pena E, Barja I, Carranza J. Social environment with high intrasexual competition enhances the positive relationship between faecal testosterone and cortisol metabolite levels in red deer. Mammalian Biology (2021) 101: 207–215.
Feces	European Bison - *Bison bonasus*	Parasites, age, sex, visitor number	After defecation, no specific time, fresh samples	Klich D, Łopucki R, Gałązka M, Ścibior A, Gołębiowska D, Brzezińska R, Kurszewski B, Kaleta T, Olech W. Stress hormone level and the welfare of captive European bison (Bison bonasus): the effects of visitor pressure and the social structure of herds. Acta Veterinaria Scandinavica (2021) 63: 24.
Feces	Elk - *Cervus canadensis*	Temperature, percipitation, hunting	No specific time, fresh samples	Pero EM, Chitwood MC, Hildreth AM, Keller BJ, Millspaugh RJ, Sumners JA, Hansen LP, Isabelle JL, Breuner CW, Millspaugh JJ. Physiological acclimation of elk during population restoration in the Missouri Ozarks, USA. Conservation Physiology (2022) 10: coac009.
Feces	Roe deer - *Capreolus capreolus*	Immunity parameters	Directly from rectum	Carbillet J, Hollain M, Rey B, Palme R, Pellerin M, Regis C, Geffré A, Duhayer J, Pardonnet S, Debias F, Merlet J, Lemaître JF, Verheyden H, Gilot-Fromont E. Age and spatio-temporal variations in food resources modulate stress-immunity relationships in three populations of wild roe deer. General and Comparative Endocrinology (2023) 330: 114,141.
Feces	Chamois - *Rupicapra rupicapra*	Forage quality and season	10 min after defecation	Corlatti L, Palme R, Valencak TG, Gomez KM. Season-dependent impact of forage quality on stress in alpine chamois. Ecology and Evolution (2023) 13: e10045.
Feces	Red deer - *Cervus elaphus*	Weather condition, seasonality, tourism, nutrition	No longer than 1 h	Gort-Esteve A, Carbajal A, López M, Manteca X, Ruiz-Olmo J, Riera JL. Faecal cortisol levels in a wild Iberian red deer population are best explained by prior weather conditions. Journal of Zoology (2024) 322: 375–385.

Table 1 (continued)

Matrices	Species	Variables	Time of sample collection	References
Feces, hair	Reindeer - *Rangifer tarandus*	Parasites- between infected group and noninfectet group	No specific time, fresh samples	Carlsson AM, Mastromonaco G, Vandervalk E, Kutz S. Parasites, stress and reindeer: infection with abomasal nematodes is not associated with elevated glucocorticoid levels in hair or faeces. Conservation Physiology (2016) 4: cow058.
Feces, hair	Mountain goat - *Oreamnos americanus*	Age, sex, reproductive status	No specific time, fresh samples	Dulude-de Broin F, Cote SD, Whiteside DP, Mastromonaco GF. Faecal metabolites and hair cortisol as biological markers of HPA-axis activity in the Rocky mountain goat. General and Comparative Endocrinology (2019) 280: 147–157.
Feces, hair	Mountain goat - *Oreamnos americanus*	Predation	Directly from rectum	Dulude-de Broin F, Hamel S, Mastromonaco GF, Côté SD. Predation risk and mountain goat reproduction: Evidence for stress-induced breeding suppression in a wild ungulate. Functional Ecology (2020) 34: 1003–1014.
Feces, Saliva	Moose - *Alces alces*	Temperature, age	No specific time, fresh samples	Thompson DP, Crouse JA, Jaques S, Barboza PS. Redefining physiological responses of moose (Alces alces) to warm environmental conditions. Journal of Thermal Biology (2020) 90: 102,581.
Hair	Red deer - *Cervus elaphus*	Sex, age, population densities, environmental conditions	No specific time	Caslini C, Comin A, Peric T, Prandi A, Pedrotti L, Mattiello S. Use of hair cortisol analysis for comparing population status in wild red deer (Cervus elaphus) living in areas with different characteristics. European Journal of Wildlife Research (2016) 62: 713–723.
Hair	Roe deer - *Capreolus capreolus*	Rutting season	After death, no specific time	Ventrella D, Elmi A, Barone F, Carnevali G, Govoni N, Bacci ML. Hair testosterone and cortisol concentrations in pre-and post-rut roe deer bucks: Correlations with blood levels and testicular morphometric parameters. Animals (2018) 8: 113.
Hair	Red deer - *Cervus elaphus*	19 minerals	After death, no specific time	Montillo M, Caslini C, Peric T, Prandi A, Netto P, Tubaro F, Pedrotti L, Bianchi A, Mattiello S. Analysis of 19 minerals and cortisol in red deer hair in two different areas of the stelvio national park: A preliminary study. Animals (2019) 9: 492.
Hair	White-tailed deer - *Odocoileus virginianus*	Age, sex	After death, no specific time	Potratz EJ, Brown JS, Gallo T, Anchor C, Santymire RM. Effects of demography and urbanization on stress and body condition in urban white-tailed deer. Urban Ecosystems (2019) 22: 807–816.
Hair	American Bison, moose, white-tailed deer - *Bison bison*, *Alces alces*, *Odocoileus virginianus*	Predation	After death, no specific time	Shave JR, Derocher AE, Cherry SG, Thiemann GW. Chronic stress and body condition of wolf-killed prey in Prince Albert National Park, Saskatchewan. Conservation Physiology (2019) 7: coz037.
Hair	Moose - *Alces alces*	Infestation of deer keds	After death, no specific time	Madslien K, Stubsjøen SM, Viljugrein H, Ytrehus B, Solberg EJ, Kapronczai L, Mysterud A, Godforid J, Janz DM, Cattet, M. Hair cortisol concentration and body mass in moose (Alces alces) infested with deer keds (Lipoptena cervi). Journal of Wildlife Diseases (2020) 56: 687–692.
Hair	Moose - *Alces alces*	human disturbance, ungulate competition, large carnivore density, ambient temperature	After death, no specific time	Spong G, Gould NP, Sahlén E, Cromsigt JP, Kindberg J, DePerno CS. Large-scale spatial variation of chronic stress signals in moose. Plos One (2020) 15: e0225990.
Hair	Roe deer - Capreolus capreolus	Sex, red deer population density, season	No specific time	Franchini M, Peric T, Frangini L, Prandi A, Comin A, Rota M, Filacorda S. You're stressing me out! Effect of interspecific competition from red deer on roe deer physiological stress response. Journal of Zoology (2023) 320: 63–74.
Hair	Reindeer - *Rangifer tarandus*	Sex, season, body position of hair samples	No specific time	Rakic F, Fernandez-Aguilar X, Pruvot M, Whiteside DP, Mastromonaco GF, Leclerc LM, Jutha N, Kutz SJ. Variation of hair cortisol in two herds of migratory caribou (Rangifer tarandus): implications for health monitoring. Conservation Physiology (2023) 11: coad030.

Table 1 (continued)

Matrices	Species	Variables	Time of sample collection	References
Urine	Mule deer - *Odocoileus hemionus*	Season, population density, snow depth, suplementar feeding	After urination, no specific time	Saltz D, White GC. Urinary cortisol and urea nitrogen responses to winter stress in mule deer. The Journal of Wildlife Management (1991) 1–16.
Urine	Black-Tailed deer - *O. heinosus columbianus*	Season, captive vs. free-ranged	No specific time	Parker KL, DelGiudice GD, Gillingham MP. Do urinary urea nitrogen and cortisol ratios of creatinine reflect body-fat reserves in black-tailed deer? Canadian Journal of Zoology (1993) 71: 1841–1848.
Urine	Elk - *Cervus canadensis*	Winter progression, nutritional stress	After urination, no specific time	White PJ, Garrott RA, Heisey DM. An evaluation of snow-urine ratios as indices of ungulate nutritional status. Canadian Journal of Zoology (1997) 75: 1687–1694.
Urine	White-tailed deer - *Odocoileus virginianus*	Supplemental dietary tannins	No specific time	Chapman GA, Bork EW, Donkor NT, Hudson RJ. Effects of supplemental dietary tannins on the performance of white-tailed deer (Odocoileus virginianus). Journal of Animal Physiology and Animal Nutrition (2010) 94: 65–73.

The number of studies that used urine as a matrix to measure glucocorticoid levels was four of the 77 studies found. The ungulate species from which urine samples were collected to analyze glucocorticoid levels were elk, black-tailed deer, white-tailed deer, and mule deer, and all animals from which urine was collected were alive.

In captivity, urine was collected from animals in cages, and they remained in the cage until they urinated, or they were kept in enclosures with steel gate floors where the urine flowed through a stainless steel pipe system and was then collected [52, 53]. Parker et al. [54] collected urine samples from captive black-tailed deer, but did not describe how this was done. Animals living in the wild were fitted with radio signals and tracked down in winter for urine sampling. The researchers observed the animals from a distance of five to 30 m and collected the most concentrated part of the urine-soaked snow [31]. Collecting urine when there is no snow can pose a problem for researchers because the ground absorbs the liquid. In the laboratory, the snow-soaked urine was thawed at 22 °C, mixed thoroughly and frozen in plastic tubes [31, 55]. No detailed information was given on the preparation of the urine samples. Only White et al. [31] mentioned extraction with triethylammonium formates prior to RIA. No detailed information was provided on the preparation of urine samples and the timing of urine sample collection after urination. In all four studies, the creatinine concentration was quantified before the results of the assays were evaluated.

Only two studies on glucocorticoid levels in the saliva of wild ungulates were found in the database. Both studies were conducted not only with salivary cortisol, but also included other matrices from which hormone levels can be extracted. In one study feces and saliva were used, in the other study all matrices mentioned above were used. Saliva was collected from Alpine ibex and moose kept alive in enclosures. Alpine ibex saliva was collected only once with swabs (Salivette®, Sarstedt AG & Co, Nümbrecht, Germany) and moose saliva was collected with synthetic swabs (SalivaBio Chil dren's Swab, Salimetrics LLC, Carlsbad, CA, USA) every morning from May 31 to August 20. The moose approached with 500 g of food to elicit the salivary response. The samples were frozen at -20 °C. No detailed information on the preparation of the saliva samples was provided.

In 15 studies, hair was used as the matrix for the quantification of glucocorticoids, with nine of the studies focusing only on hair and the other six studies using other matrices in addition to hair. The hair for the analysis of cortisol levels was plucked from red deer, roe deer, reindeer, white-tailed deer, moose, Alpine ibex, mountain goat and American bison. In 10 studies, researchers used hair samples from dead animals (culled, killed by predators or run over) and in four studies hair samples were

taken from live animals. In 12 studies the animals lived in the wild and in three studies the animals were kept in enclosures (Table 1). In none of the studies was the time of hair sampling specified, with the exception of one study conducted on the Alpine ibex, which stated that the hair samples were taken immediately after the animals were restrained. In this study, all matrices described in this review were used [17].

In nine studies, the researchers acknowledged that hair growth rate should be taken into account when interpreting glucocorticoid levels determined from hair samples. Of these nine studies, only one knew the exact timing of the growth rate, as the researchers shaved a section of hair before the experiment and shaved it again after the experiment and redetermined the exact number of days of hair growth rate [56]. The other seven studies only estimated the active hair growth period, as the researchers claimed that the glucocorticoids passively diffuse during the active growth phase and stay incorporated in the hair during the rest phase [57].

Variables influencing hormone levels

The variables tested for their influence on glucocorticoid levels in the different matrices were categorized into three groups: anthropological, environmental and biological. Anthropological variables included all human-mediated stressors acting directly on the animals (handling/restrain in captured animals, hunting and wounding in harvested animals, etc…) or on their habitat (presence of livestock, urbanization, infrastructures, etc…). Environmental variables were mainly based on season, climatic conditions and circadian rhythm. Biological variables included sex, age, social ranking of the animals, physiological status and predation (Table 1).

The variables that influenced the levels of glucocorticoid metabolites in the feces examined in all studies were diverse and some studies considered all three types of variables. Anthropological variables included hunting, chemical immobilization and the presence of livestock, urbanization such as roads and road noise, and wind turbines [58–60]. Fecal samples collected in national park regions were used to compare cortisol levels with tourism influences such as visitor numbers, hikers, and snowmobile activity [61–63]. Environmental variables were mainly based on season, temperature, precipitation, water level, snow depth, winter patterns and circadian rhythm. Biological factors examined in the studies reviewed included animal sex and age, social rank, group and harem size, reproductive status, rutting season, testosterone levels, antler size, food quality and availability, parasites and predation [27, 63–66]. Some studies even investigated competition with other wild ungulates [67].

The researchers used urine to investigate whether the season, the course of winter, snow depth and population density had an influence on glucocorticoid levels. They also investigated whether dietary stress was present and whether supplementary feeding of captive animals and tannins in the diet had an impact on stress levels. Three of the four studies used urine from captive animals and one study used urine from free-ranging animals. In one study, the cortisol levels of captive and free-ranging ungulates were compared and no significant difference was found between the two groups studied (Table 1) [54].

Saliva was used for testing if environmental and biological variables such as: season, ambient temperature, sex and age of the animals influenced salivary cortisol levels [17, 68].

The variables tested that influence glucocorticoid levels in hair were anthropological, environmental, biological and non-categorical variables. Anthropological variables tested for their impact on cortisol levels included human disturbance and hunting [69]. Among the environmental variables we included the season and ambient temperatures, and among the biological variables we included sex and age of the animals in question, pregnancy of females, parasite infestation, population density, competition with other ungulates, predation, and density of large carnivores [56, 57, 70]. One study investigated whether hair body position had an effect on glucocorticoid levels, and one study investigated whether minerals (19 minerals) also had an effect on cortisol levels [71, 72]. We did not categorize these two variables.

The anthropological influences on the HPA axis of ungulate species belonging to the families *Cervidae* and *Bovidae* were investigated in a total of 21 studies, most of which were conducted on cervids, more precisely on red deer and roe deer. A large number of studies also compared anthropological influences on chamois. Anthropological variables consistently influenced blood glucocorticoid levels in wild *Cervidae* and *Bovidae*, and 73% of the reviewed studies confirmed this positive correlation with their fecal metabolites. Only one study linked anthropological variables to glucocorticoid levels in hair and showed no correlation.

Season was the most commonly studied environmental variable in *Cervidae* and *Bovidae* and its influence on glucocorticoid levels was confirmed in 50% of the studies that used blood as a matrix and in 80% of those that measured their metabolites in the feces. It is noteworthy that feces were the most commonly used matrix to determine seasonal influences on target species. In most studies examining the seasonal trends of glucocorticoids, the researchers found that the increased concentrations of the hormones and their metabolites occurred in the cold seasons, but in some cases the increased concentrations were found in the summer and warmer seasons. This was the case for alpine ibex, elk, bighorn sheep, reindeer and roe deer [17, 73–76]. Chamois and white-tailed deer

mostly showed elevated cortisol levels in the cold season, but in the study by Delgiudice et al. [77] and Hadinger et al. [78] the seasonal trend was reversed, with the higher levels occurring in the warmer seasons.

In three studies on three different animal species (chamois, elk, red deer), snow depth was positively correlated with fecal glucocorticoid metabolites [62, 79, 80]. Other environmental factors tested for their influence on fecal hormone metabolites were temperature, solar radiation and precipitation. Precipitation had no effect on hormone metabolites, but the effect of temperature varied among chamois. In one study, fecal metabolites were shown to increase with increasing temperature, while in another study no effect of temperature was found [27, 80].

The influence of individual variables, as age and sex, was also frequently studied. In almost all studies, sex had no effect on glucocorticoid levels, but 29% of studies using blood and 22% of studies using hair showed otherwise. The age of the animals tested in the reviewed studies showed an effect on glucocorticoid levels in blood (67%) and on its metabolites in feces (64%), while glucocorticoids in hair could not be linked to age as an influencing factor, as 71% of the studies showed that age did not affect hormone levels. A common pattern was observed with higher values in older animals than in younger animals. No study linked age and sex with the hormone concentrations in the urine.

Not surprisingly, almost all studies (four out of five in total and in different matrices: blood, feces and hair) confirmed that presence of predators was positively correlated with the level of glucocorticoids and of their metabolites, with only one study using elk feces not confirming these findings. Four studies compared parasite infestation with glucocorticoid metabolites in feces and one study compared it with glucocorticoid levels in hair. 50% of the studies that used feces and one study that used hair proved that parasites had an impact on the animals' glucocorticoid concentrations.

Influences on reproduction variables such as the rutting season, testosterone levels, social rank, harem size, gestation and lactation were also tested for a stressful effect on the organism of the different animals. In four out of five studies, the rutting season was shown to have an effect on the HPA axis. Testosterone levels had an effect on fecal glucocorticoid levels and were positively correlated in both studies in which this variable was tested [66, 81]. Fecal metabolites in pregnant red deer were elevated in the late stages of gestation, while glucocorticoid levels in hair did not increase with pregnancy in red deer and white-tailed deer [51, 64, 72]. In two studies, the researchers used red deer feces and mountain goat hair to investigate whether lactation has an effect. They found that glucocorticoid concentrations in feces varied according to lactation status at the time of sampling, while glucocorticoid concentrations in hair did not vary [65, 82]. Harem size was tested in one study on red deer feces only, and the deer with larger harem sizes had higher concentrations of glucocorticoid fecal metabolites [64]. Social rank was also tested in one study in white-tailed deer, and fecal glucocorticoid concentrations were not affected by the social rank of the animals [83].

In a study using elk feces to measure hormone metabolites, elks from larger groups were found to have lower fecal glucocorticoid levels [84]. In contrast, other study that used hair samples of red deer found that higher population density was linked to higher glucocorticoid levels [85].

Diet quality and nutrition were used as variables in three studies, with two studies using feces and one study using urine as a matrix to determine the results. One study that used feces as a matrix compared fecal samples from roe deer living in pine and oak forests and showed that stress levels were higher in roe deer living in pine forests [86].

Table 1. Variables affecting glucocorticoid concentrations in all matrices and timing of sample collection (Please insert Table 1 here).

EIA or RIA

Of the total proportion of studies that used EIA as the method of choice, 26.6% used feces as the matrix, 26.0% used combined matrices, 20.2% used hair, 18.2% used blood, and 9.1% used urine to determine glucocorticoid concentrations. Of the total proportion of studies that used RIA as the method of choice, 12.1% used feces as the matrix, 7.9% combined matrices, 24.6% hair, 13.8% blood, and 41.5% urine. Of the studies that used both EIA and RIA, 69.6% used more than one matrix to analyze cortisol levels and the other 30.4% used blood. In the studies that used analyzes other than EIA or RIA, 20.6% used feces and 79.4% used blood for cortisol determination (Fig. 3).

The most commonly used analysis to quantify cortisol in almost all matrices was the EIA method, except for urine, where the most commonly used analysis to quantify cortisol concentration was the RIA method. Three of the 4 papers that used urine as a matrix performed RIA analyses and one performed EIA. Four papers that we found and included in our review did not use RIA or EIA to quantify cortisol, but used NIRS, LC-MS/MS, HPLC-MS/MS, and Kodak Amerlite. One study analysed blood samples from reindeer, mentioning only that commercially available kits were used and which companies produced them, without specifying the analytical method used [73].

More than half of the studies (30 studies) in which EIA was used as an analytical method validated the method itself for the species in question or used it because it had

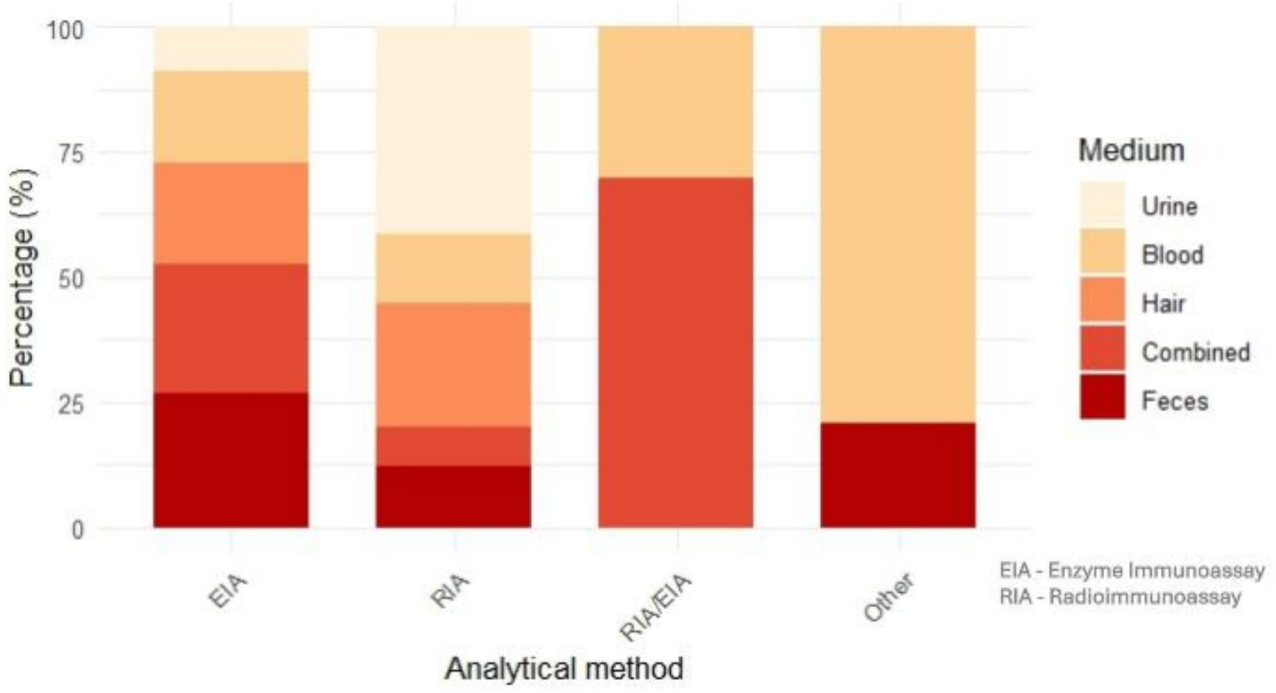

Fig. 3 Bar charts representing percentages of analyses for every matrix (combined meaning more than one matrix used in one study). Bar "Other" include analyses other than EIA and RIA, specifically Kodak Amerlite, HPLC-MS/MS, LC-MS/MS and NIRS. RIA/EIA chart are studies that did both RIA and EIA analyses

previously been validated for use in the species in question [80, 82, 84, 86–98]. In 19 studies, the EIA method was not validated [75, 76, 78, 99–105]. Twenty-one studies used RIA as the analytical method of choice. Eleven of them did not validate the method, while 6 of them validated the method and 4 of them mentioned that it had been previously validated for the target species [77, 106, 107]. Two papers used both of the above analytical methods and both were not validated. Of the papers that used analyses other than EIA and RIA, the methods were not validated [47, 108, 109]. One exception was the study that used NIRS analysis for cortisol levels, in which the researchers validated the NIRS analysis method [110]. Finally, the study in which the assay used was not specified did not provide information on validation of the method [73].

Of the 40 studies that validated the methods, 18 performed the validation procedure themselves. Validation of the RIA methods on urine samples was performed using parallelism, quantitative recovery and intra-assay coefficients of variation for cortisol [52, 56]. Validation of the RIA method for quantification of glucocorticoid metabolites in fecal samples was performed using the ACTH challenge test, parallelism, recovery of the exogenous analyte, intra- and inter-assay precision, and finally assay sensitivity. The EIA method for quantitative measurement of glucocorticoid metabolites in feces was validated with high performance liquid chromatography (HPLC), ACTH challenge, parallelism, linearity recovery, intra- and inter-assay precision test, repeatability test and assay sensitivity. The method was also validated in two studies using EIA for the quantification of glucocorticoids in hair. The researchers used ACTH challenge, HPLC analysis, parallelism, and intra- and inter-assay coefficients of variability. All studies in which the researchers validated the method themselves confirmed that the assays are suitable for use in quantitative measurements of glucocorticoids and their metabolites.

Trend analysis

The number of peer-reviewed scientific publications on stress in wild ungulates increased by 100% between 1979 and 2024. From the year 2000, which was taken as the median year, and up to 2024, the number of publications on cortisol levels in wild *Cervidae* and *Bovidae* on the targeted geographical regions increased by 500%. The number of publications was relatively stable until the year 2007, when the increase in published papers began. Since then, the number of publications has been increasing, with the most papers published in 2020, when the number of published papers on this particular topic amounted to 10 (Fig. 3).

Discussion

After reviewing the available scientific papers on the quantification of glucocorticoids and their metabolites in different biological matrices in the family *Cervidae* and *Bovidae*, we showed that research efforts covered

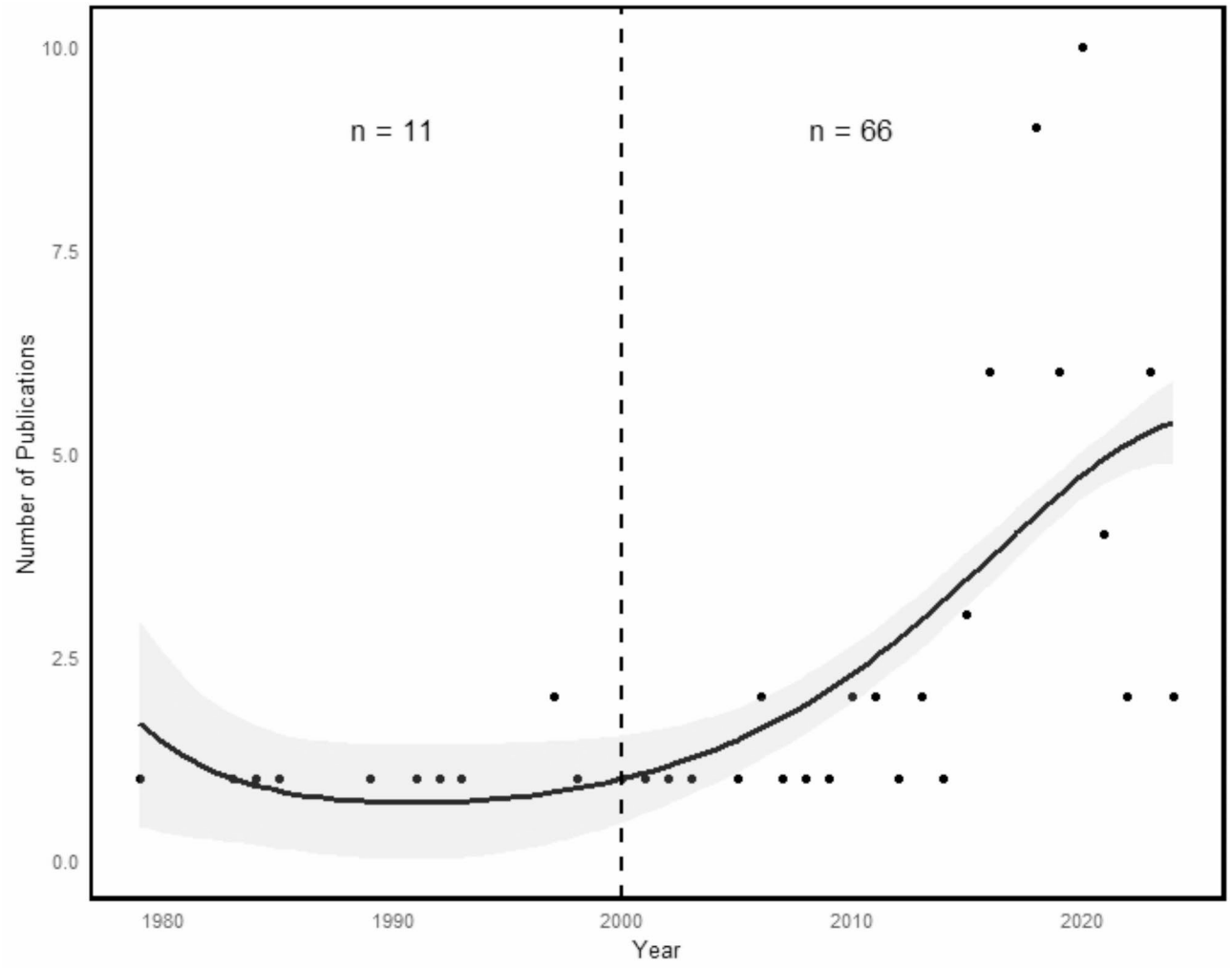

Fig. 4 Number of peer-reviewed scientific papers on cortisol levels in *Cervidae* and *Bovidae*, between 1979 and 2024. The fitted line shows the expected response of a 3rd level polynomial autoregressive Poisson model; the shaded area indicates the 95% confidence level; the dashed line separates the first two decades from the last two decades (midyear 2000 was used as a midpoint)

approximately 42–45% of the species present in the European and North America continents, with an even higher coverage for *Cervidae* species in North America (75% of the species).

On the North American and European continents, there are numerous species of wild animals that live in the wild or are kept in captivity, farms, etc. Some are native and others have spread by natural means or directly and indirectly by anthropogenic dispersal [111]. Nevertheless, there is a wide range of animals living in the wild on both continents, as well as species belonging to the *Cervidae* and *Bovidae* families [112]. As the literature search yielded scientific papers on 17 taxa for both continents, it is evident that there is much more potential for research on other animal species belonging to the targeted families, giving us more insight into the coping mechanisms for stressful situations that the wild provides for animals. In addition, only one or a few studies on glucocorticoid levels were conducted in some species studied, which gives us little insight into the animals stress responses and opens up further research opportunities. We would not attribute the difference in the number of papers on certain species to the accessibility of the terrain in which they naturally live, as there are many papers on chamois living in terrain that are harsh and difficult for researchers to sample. Among the most studied species in our work are red deer and chamois. The red deer is one of the most abundant and widespread deer species in Europe and is of great economic, cultural and ecological importance [113]. The chamois is the most common mountain ungulate in Europe [96]. The difference in the number of studies on the individual species could be due to the abundance and distribution of each species.

In the reviewed articles blood samples were taken from animals culled during the hunting season, shortly after shooting. Blood collection was described as quick and

easy. The only problem encountered in obtaining blood samples from culled animals was contamination of the blood with rumen, stomach or intestinal contents. The contaminated blood was discarded and not used for the studies [22, 113]. When collecting blood samples from culled animals, researchers should examine the animal and carefully collect the blood samples and ensure that they are not contaminated with intestinal or other contents to obtain accurate results. Bubenik et al. [114] collected blood samples from live animals via cannulas inserted into anesthetized bucks. Bartoš et al. [49] used a crush to collect blood from red deer. Trondrud et al. [109] captured wild reindeer with snowmobiles and slowly steered the animal in the desired direction, and when the animal was close enough, they captured it with nets. This shows that blood sampling from live animals is more labor intensive and expensive. When planning on using blood as a matrix for cortisol analysis, it is necessary to consider that the capture of the animals and the trauma of shooting and wounding trigger an acute stress response and activate the HPA axis, thus affecting the final results of glucocorticoid levels in the blood [45, 109]. When using blood as a matrix for the quantification of glucocorticoids in live or culled animals outside the hunting season, special ethical approvals must be obtained from the relevant institutions, which can be time-consuming. Researchers should take this into account when planning to use similar experimental designs.

Understanding the dynamics of the circulation of bound and unbound glucocorticoids is essential to accurately interpret the differences in concentrations in the different matrices. When measuring glucocorticoid levels in the blood, researchers should indicate whether the target of their analysis is the total, bound or unbound hormone. Glucocorticoids must interact with a receptor in order to exert a physiological effect. If hormone concentration is increased due to chronic stress events, the number of hormone receptors decreases, and larger amounts of unbound hormones remain in the blood [25]. As can be seen from the results, most studies using blood did not include an extraction procedure prior to quantification of glucocorticoids, i.e. they measured the bound and unbound hormone. Only unbound glucocorticoids are biologically active and should therefore be the target of analysis. Researchers should consider extracting the free part of the hormone before performing the quantification analysis. Extracting the free part of the hormone before the analysis requires a few more steps and takes a little more time, but the results are more specific and accurately reflect the effects of the stressful events to which the animals are exposed [115].

Kastelic et al. [17] used blood, feces, hair and saliva as matrices and found that the highest mean value of the hormone cortisol was found in blood and the lowest in saliva. This information can help researchers decide which matrix to use for their research. To minimize the invasiveness of blood sampling, there are other ways to collect blood samples from animals by kissing bugs and leaches. A comparison of the concentration of glucocorticoids in samples obtained by venipuncture, kissing bugs and leeches showed that the concentrations of hormones were highest in serum samples obtained by venipuncture and lowest in samples obtained by kissing bugs. No statistically significant differences were found between the serum concentrations obtained by venipuncture and with leeches. Blood collection from kissing bugs is a delicate procedure in which needles are used to collect small amounts of blood. If the operator is careless, the contents of the bug's gastrointestinal tract can contaminate the sample and falsify the results [17]. These methods still need further research, but they have the potential to be used as alternative methods to venipuncture blood sampling.

Feces were used to determine glucocorticoid metabolites in almost half of the papers examined, making feces the most commonly used matrix for analyzing glucocorticoid levels in wild ungulates. One reason for the frequent use of this matrix could be that the collection of feces is not associated with stress for the animals and that it provides information on long-term hormone levels [24, 28, 50]. For the collection of feces from wild animals, it is necessary to collect fresh feces, as the glucocorticoid metabolites are unstable and decrease over time [30]. In many papers we reviewed, only fresh fecal samples were collected in plastic bags and stored in a portable refrigerator until the field work was done, usually a few hours, and after the field work the samples were stored in a freezer at -20 °C [61, 79, 116, 117]. Sometimes it was also possible to freeze the fresh samples immediately in the field [118]. Creel et al. [62, 119] stored the fecal samples at -80 °C. Researchers interested in studying stress variation in wildlife are advised to collect fresh fecal samples and store them in the freezer as soon as possible to avoid misleading interferences [30]. Aside from a pair of gloves, plastic bags, a portable refrigerator, and a good physical condition to walk in the field, there is not much to prepare for field fecal sampling. Prior to the analysis of fecal glucocorticoid metabolites, the fecal samples were thawed and homogenized. Some used phosphate-buffered saline, others methanol or ethanol for the extraction of cortisol metabolites from feces [48, 81, 120, 121]. The simplest preparation of samples for the extraction of cortisol metabolites from feces was described by Palme et al. [122]. The amount of 0.5 g of well homogenized fecal sample is extracted with 5 ml of 80% methanol and further diluted with assay buffer and then analyzed with the EIA kit [27, 122]. When planning sample preparation

for analysis, it is best to investigate the most appropriate sample preparation for the analysis to be performed.

The distribution of glucocorticoid metabolites in feces is uneven, and they are not randomly distributed across the samples. Hormone metabolites can be localized in the sample creating "hot spots" that can alter the results of the analysis. This can be a greater problem with large fecal masses than with smaller fecal samples. This problem can be solved by homogenization of the samples [25, 74]. However, in a large number of studies on the quantification of glucocorticoid metabolites in feces, homogenization of the samples prior to analysis was not performed. If possible, the entire pile of the sample should be collected and mixed well before any analytical procedures are performed to avoid localization of glucocorticoid metabolites.

Fecal glucocorticoid metabolites, unlike blood levels, are less influenced by the episodic fluctuations of hormones or by the pulsatility of hormone secretion. For this reason, some researchers argue that fecal glucocorticoid metabolite levels more accurately represent an animal's endocrine profile than blood samples [10, 121].

As far as urine sampling is concerned, it is easier to collect urine from captive animals than from wild animals. The few papers in which urine was used as a matrix for the quantification of glucocorticoids can be attributed to the difficulty of collecting urine in the wild. There is an opportunity to explore alternative methods of collecting urine samples from wildlife that can be used in further research. As our results show, the only method of collecting urine from *Cervidae* and *Bovidae* to date is the collection of snow-soaked urine, which limits research to the winter months and therefore does not provide good insight into stressful times of the year [31].

When performing analyses on urine it is necessary to measure creatinine levels, as creatinine is used as an index of urine production per unit of time. Its excretion over a 24-hour period is relatively constant, which is why it is used to calculate differences in urine concentration and to standardize values in urinary glucocorticoid level analyses [52].

The excretion patterns of glucocorticoids in the urine of wild animal species still need to be investigated in more detail. For example, in a study with golden snub-nosed monkeys *Rhinopithecus roxellana*, urine and feces were collected before and after stressful events. The peak level of glucocorticoids in urine was detected within 5 h after the stressful event [123]. To effectively monitor glucocorticoid concentrations in the urine of wild ungulates, more studies need to be conducted on this topic. In addition, samples should be taken at regular intervals to determine the species-specific patterns of cortisol excretion, as the concentration of hormones in urine reflects glucocorticoids in the blood [123].

Salivary cortisol represents the parameter of cortisol that is unbound in the blood and can cross membranes, making salivary cortisol a tool for monitoring acute stress. Obtaining saliva samples from wild animals is a challenge for researchers. In the studies reviewed, samples were collected from animals kept in enclosures, so sampling was relatively easy [17, 31]. Good and cost-effective collection methods need to be developed and validated for the collection of saliva from wild animals, not only for glucocorticoid research but also for other types of research involving saliva. In a small pilot experiment in Bulgaria, different types of swab baits were used in supplemental feeding of wild ungulates and good results were obtained, although the techniques still need further development [124].

The hair of hunted animals, road-killed animals and of animals killed by predators can be easily collected in the field. The hair can be cut, plucked or shaved, or a piece of skin with hair can be obtained from dead animals [51, 70, 72, 125]. For animals kept in enclosures, it is also easy to collect hair by cutting a tuft of hair right next to the skin [17]. Davenport et al. [126] recommended washing the hair strand in 5 ml isopropanol to minimize the risk of extracting cortisol from the hair. Washing the hair with isopropanol also ensures the removal of sweat and sebum steroids. After washing the hair samples with isopropanol or methanol, the hair was usually ground to a fine powder, centrifuged and incubated overnight with methanol for cortisol extraction. The methanol was allowed to evaporate before analysis [50, 69, 72, 127]. The glucocorticoid concentrations in the hair reflect the cumulative or chronic stress to which the animals were exposed over weeks to months [128].

The analysis glucocorticoid concentrations in hair is a relatively new method for determining stress levels in wild ungulates, as the 2 oldest papers we found date back to 2016 [56, 85]. As mentioned above, since 2016, 15 papers have used hair as a matrix for determining stress levels in wild ungulates. The increase in the number of papers can be attributed to the relative ease of collecting, storing and preparing hair samples for analysis. As the method is relatively new, more detailed studies should be carried out to test its accuracy. Theoretically, hair samples should represent the activity of the HPA axis throughout hair growth, thus making the level of glucocorticoids in hair reflect an individual's HPA activity during the hair growth phase [57, 71]. According to this theory, the hair growth rate should be accurately calculated for each individual. Most studies do not measure hair growth rates, as this can be very difficult in wild animals. In addition, recent studies question the reliability of glucocorticoids in hair as a marker of past stress [129]. Colding-Jørgensen et al. [129] investigated the accumulation of glucocorticoids in the hair of rats over time. They

treated rats with 50 mg/ml corticosterone emulsion over a period of 7 days. The glucocorticoid concentration in the hair increased with the treatment, but after the end of treatment, the glucocorticoid concentration in the rats' hair samples decreased [129]. This result may suggest that glucocorticoids in hair do not provide an accurate pattern of glucocorticoid secretion over time and are thus a marker of past stress, but primarily reflect the concentrations present at the time of collection. Indeed, the method needs to be studied more thoroughly in other animals and researchers should be cautious in interpreting the results.

Furthermore, Romero and Beattie [130] argue that the levels of glucocorticoids in other matrices are not always representative of levels in blood. They argue that the time lag in the uptake of glucocorticoids and their metabolites into other matrices play an important role in the correlation with blood. They also propose that there is a species-specific time delay in the uptake of glucocorticoids. To further develop this theory in wild ungulates, it is essential to investigate the correlation between the concentrations of glucocorticoids in blood and other matrices [130].

It should be noted that there were significant differences in a study in which both guard hair and undercoat were used to quantify glucocorticoid levels in the hair. The glucocorticoid concentration in the hair was much higher in the guard hair than in the undercoat [82]. This was also shown in the results of the study by Macbeth et al. on brown bears (*Ursus arctos*) [131]. The difference could be due to variation in hormone incorporation in the hair, as this can vary according to the chemical and physical properties of the hair types. Therefore, researchers considering using hair as a matrix for hormone detection should be aware of the differences in hormone levels in different hair types and stick to one hair type when analysing hormone levels, as the different levels of glucocorticoids could affect the predictive power of the correlation and thus provide inaccurate results [82]. From the literature search there were found only two studies on reindeer that tested if the hair from different parts of the body had different glucocorticoid levels and in both studies the hair obtained from different parts of the body did not have significant differences in glucocorticoid concentrations. And according to these results, researchers need not worry about the positioning of the hair, as it does not play an important role. However, more detailed studies need to be carried out on different animal species to be sure [56, 125].

Glucocorticoids are a good indicator of whether animals have been exposed to stressful situations, which is why they are used in ecological and conservation studies. As the number of studies on glucocorticoid levels in wildlife increase, new insights into the glucocorticoid responses of wildlife are emerging, as high acute elevations in glucocorticoid levels do not always indicate animals in distress and, conversely, low glucocorticoid levels do not always mean that animals are not under stress [130]. When interpreting the results, researchers should pay attention to the season in which the sampling was performed, regardless of the matrix used for quantification of the glucocorticoid levels or their metabolites. This is very important for the stress values, as different biological processes take place in the animal organisms. For example, mating seasons, estrus and pregnancy in females as well as lactation periods potentially increase stress hormones [10, 76, 85].

Variables influencing hormone levels

The large number of studies performed on red deer leaves a large gray area in the knowledge of anthropological influences on other species belonging to the same families. There are many research opportunities to be explored that can help us learn more about the coping mechanisms of *Cervidae* and *Bovidae* in relation to human presence. It is interesting to note that fecal glucocorticoid metabolites were not elevated in Alpine ibex after they were chemically immobilized and treated for the collection of biometric data and biological samples. Fecal samples collected before and during immobilization were compared with samples collected twice on five consecutive days after immobilization, and glucocorticoid metabolites did not differ [59]. In contrast, snowmobile trapping increased glucocorticoid levels in the blood of reindeer [109]. Chemical immobilization of wild animals prior to capture had no effect on the stress response measured in feces, while the more robust method of capture resulted in an acute stress response in the animals. Researchers who choose to use blood as a matrix to determine glucocorticoid levels in live wildlife should consider the method of immobilization and capture of the animals.

The difference in reactions to an increased number of tourists was observed in red deer and chamois feces. In other studies, fecal hormone metabolites of the same species were elevated at higher tourist numbers [61, 93], but in one study examining both species, tourist numbers had no effect on fecal glucocorticoid metabolites [80]. The difference could be due to the different habituation of the animals to the disturbance by tourists. However, more detailed studies should be carried out to find out why some animals react differently to the increased presence of tourists. In addition to anthropological disturbances, hunting and trauma affected blood glucocorticoid concentrations and their metabolites in feces, with the exception of a study conducted on elk, which found that fecal glucocorticoid metabolites did not increase with hunting pressure [84]. It is to be expected that

hunting is stressful for wildlife, further studies are thus needed to confirm this finding.

The effect of the season on the levels of glucocorticoids and their metabolites has been studied using all matrices. The impact of the season was mostly tested on red deer, white tailed deer and chamois, resulting in small numbers and even no studies performed on other species of the targeted families testing seasonal trends of glucocorticoid levels and their metabolites. This leaves us with very little information on the seasonal trends of stress hormones and their metabolites in wild ungulates and more research could be performed to fully understand the coping mechanisms of wild ungulates during season changes. We observed a general pattern in which most animals had higher glucocorticoid metabolite levels in the colder seasons than in the warmer seasons. Elk, bighorn sheep, alpine ibex, reindeer and roe deer were an exception and showed increased concentrations in the warmer seasons [17, 73–76]. It is possible that there are seasonal differences in glucocorticoid levels between species. Some species, such as red deer and chamois, are much better studied than other species, and we cannot say with certainty that there are specific differences in seasonal trends between species. Further studies with other species are needed to compare seasonal trends between species.

Of all the species studied, the red deer has most often shown that the season has no influence on its stress levels. As there are very few or no studies on other species, it is difficult to say with certainty whether the influence of season is common in other species.

The samples can be obtained either on an anonymous basis or in connection with the identity of the animal. The difference in glucocorticoid levels was not affected by the sex of the animals studied, but age had a major influence on the different age classes and their stress levels. Recent studies have drawn attention to individual heterogeneity while assessing the variation in glucocorticoid levels in wild animals. Neglecting the individual effect may ultimately alter conclusions and lead to misleading interpretations of the effects of different stressors [132]. This is evident in the differences in age classes and their glucocorticoid levels. When possible, animals and their samples should be identified to obtain more meaningful results and avoid sample replicability.

Much more research is needed to determine which animal species from the *Cervidae* and *Bovidae* families are more susceptible to certain biotic and abiotic factors.

As the results show, it is possible to investigate various anthropological, environmental and biological influences on glucocorticoid levels in wild ungulates. As the climate changes and the world moves towards a drier and hotter climate, wild animals have to cope with more environmental stressors such as higher ambient temperatures, droughts and food shortages [133].

EIA or RIA

EIA and RIA assays are frequently used in endocrinology because they are robust and have a high sensitivity [85]. RIA has long been considered the standard analytical method for the determination of glucocorticoid concentration, although it has some disadvantages and negative factors, such as radiation, the production and disposal of radioactive waste, and the short shelf life of the reagents used for the analytical procedure [134, 135]. Although the RIA provides very accurate results, researchers have found the EIA to be an alternative, user-friendly and safe test with high sensitivity and very accurate results [40]. The EIA test is gaining popularity in endocrinology because it is non-radioactive and does not produce hazardous waste, making it environmentally friendly. It is also less labor-intensive and faster than the RIA assay, requires less space and does not require a license. The RIA assay, on the other hand, requires specialized equipment in a lab that is classified to work with radioactive material, which puts researchers' health at risk. EIA kits are competitively priced and do not incur additional costs for the disposal of radioactive material generated when using the RIA assay. All this makes the EIA assay more economical and safer to use, making it preferable to the RIA assay [42, 136].

Analytical methods play an important role in quantifying the chemical constituents of natural or synthetic materials. To prove their accuracy, applicability and reproducibility, the method must be validated for use in all matrices and on all species [10]. Our results show that almost half of the studies did not validate the assay used in the research. Considering that validation of the assay is very important for demonstrating the accuracy of the results, it is recommended that researchers performing glucocorticoid analyzes on wild ungulates consider using validated methods or validate the method itself and make this information visible to other researchers in their work [10]. Of the total number of papers included in this review, 18 papers performed validation of the method used to quantify glucocorticoid concentrations and their metabolites in feces, hair and urine. In other studies, the validation procedure was not performed or the methods used were validated in previous studies. To confirm that the assay can accurately and precisely measure hormone levels and their metabolites, parallelism can be used to ensure that the assay maintains linearity under dilution. Recovery of the exogenous analyte is also a validation procedure that verifies accurate measurement over the entire working range of the assay [67, 120]. Intra- and inter-assay coefficients of variation are used in testing the validity of an assay to estimate the precision of the

method [137]. Assay linearity can also be used to validate assays [61]. To test the accuracy of the assays in question, the spike and recovery test can be performed [97]. To validate the assay, researchers can also use the sensitivity test. The sensitivity test measures the lowest amount of the hormone standard that can be consistently distinguished from the zero concentration of the standard [91].

ACTH challenges are used to test the biological significance and physiological relevance of an assay to quantify glucocorticoids. The ACTH challenges stimulate glucocorticoid levels in the blood, and by measuring whether the glucocorticoid levels in the matrices tested reflect the predicted changes in the blood, the method is validated [138]. In the five studies reviewed in this paper, the researchers used ACTH challenges to validate the assay method used [67, 79, 82, 88, 90]. The use of this method in free-ranging animals could have potential limitations, as the animals must be injected with ACTH to test the biological significance and physiological relevance of an assay [139]. Capturing animals and tracking the same treated animal in the wild requires a lot of time, resources and equipment.

In none of the studies did the researchers state that the methods were not suitable or appropriate for the determination of glucocorticoid quantification. All results obtained with both assays were used in further statistical analyses and provided further results on various stressors affecting glucocorticoid levels. This indicates that both assays are suitable for the quantification of glucocorticoid levels and the levels of their metabolites in animal species from the *Cervidae* and *Bovidae* families. When choosing the appropriate assay for the quantification of hormones and their metabolites, researchers should choose the assay that best suits their needs and budget, taking into account the differences mentioned above.

In addition to validation of the assay, researchers should also consider the protocols for collection, storage and preparation of samples for glucocorticoid levels analysis [10]. As mentioned above, one paper indicated that they used commercially available kits without specifying which analysis was used. The article is from 1985 and much has changed since then [73]. As we were unable to find any further information about the kit used on the basis of the manufacturer's information alone, we recommend that researchers always state which analysis method was used and not just the manufacturers of the kits used for the analysis. Companies can change their products overtime or even stop production altogether, so the paper will miss important information about the assays used.

All of the above points should be considered when deciding on an assay to quantitatively measure glucocorticoid levels. Researchers should also consider the type of sample preparation that is appropriate for their assay of choice.

Conclusion

It is possible to collect and analyze different matrices for glucocorticoid concentrations and thus gain insight into how wild ungulates cope with stressors. The choice of matrix must be made carefully and depends on the nature of the study, i.e. whether the aim of the research is to investigate acute or chronic stressors. More than half of all studies (53%) included in this review chose feces as the matrix. Considering that collecting feces does not disturb the animals and is easy to do, it is reasonable to assume that it would be the most commonly used matrix. Collecting fresh saliva from wild animals appears to be infeasible, which may be the reason why researchers avoided saliva for accessing glucocorticoid concentrations.

The analytical procedure must be demonstrably accurate and reproducible, which is why the validation of analytical procedures is necessary and important. Although validation is important, in almost half of the studies the analytical method used to determine cortisol levels was not validated. It can be seen that it is possible to use analytical methods other than EIA and RIA for the quantification of glucocorticoids, but it is recommended that the methods used are validated, as validation of the method confirms that it is fit for purpose. The RIA assay is considered the best method for ecophysiological studies, but the EIA is a very accurate, non-radioactive alternative assay for the same range of studies.

Research on stressors affecting glucocorticoid concentrations in wild ungulates is increasing. This shows that researchers are more interested in the welfare of wild animals and want to understand how wild animals cope with stressful events. The increasing number of scientific studies on cortisol levels in wildlife has made it possible to draw new conclusions about stressful events affecting wildlife, providing new insights into the biology of the animals concerned. New insights into the physiological and behavioral changes of animals as a coping mechanism for a hotter and drier environment are needed to monitor animal welfare. With new insights into the biology and ecology of wildlife comes new ideas for better wildlife management and conservation plans, as well as the development of new techniques for capturing and handling animals for research.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-025-04678-z.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Acknowledgements
Acknowledgements – The study was partially supported by the Croatian Science Foundation, project HRZZ: IP-2022-10-7502, "Wild boar fear of hunting: effects on space use, stress, and meat quality.".

Author contributions
Authors' contributions – VB conducted the database search and wrote all drafts of the manuscript NŠ and FB conceptualized the framework. All authors read, corrected, and approved the manuscript.

Funding
Croatian Science Foundation, project HRZZ: IP-2022-10-7502.

Data availability
Data is provided within the manuscript or supplementary information files.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 2 December 2024 / Accepted: 17 March 2025
Published online: 03 April 2025

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