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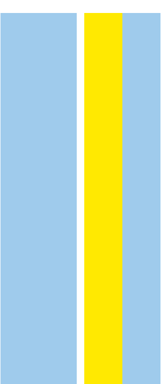
Non-invasive measurement of long-term adrenocortical activity in Iberian canivorans



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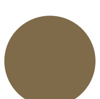

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Non-invasive measurement of long-term adrenocortical activity in Iberian carnivorans

Alexandre de Carvalho Azevedo

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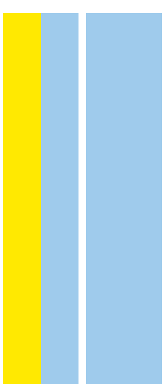

ALEXANDRE DE CARVALHO AZEVEDO

NON-INVASIVE MEASUREMENT OF LONG-TERM ADRENOCORTICAL ACTIVITY IN IBERIAN CARNIVORANS

Tese de candidatura ao grau de Doutor em Ciência Animal, Especialidade em Morfologia e Fisiologia, submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

Orientadora – Professora Doutora Liliana de Sousa
Categoria – Professora Associada
Afiliação – Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

Coorientadora – Professora Doutora Katarina Jewgenow
Categoria – Investigadora Sénior
Afiliação – Leibniz Institute for Zoo and Wildlife Research

This cumulative dissertation reports research performed between November 2016 to October 2020 at the Leibniz Institute for Zoo and Wildlife Research in the Department of Reproduction Biology under the supervision of Prof. Dr. Katarina Jewgenow, PD Dr. Martin Dehnhard and PD Dr. Jella Wauters.

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The work reported in this thesis was developed in collaboration with the Iberian lynx *ex-situ* Conservation Programme, the National Breeding Centre for Iberian Lynx, Portugal, the El Acebuche Iberian Lynx Captive Breeding Centre - OAPN, Spain, and the Department of Biology and CESAM of the University of Aveiro.

List of Publications

This thesis was based on the following publications:

Article 1: Azevedo A[1,2], Bailey L[1], Bandeira V[3], Dehnhard M[1], Fonseca C[3], De Sousa L[2] & Jewgenow K[1] (2019). Age, sex and storage time influence hair cortisol levels in a wild mammal population. *PLOS OnE*, 14(8), e0221124. https://doi.org/10.1371/journal.pone.0221124

Article 2: Jewgenow K[1], Azevedo A[1,2], Albrecht M[1], Kirschbaum C[4] & Dehnhard M[1] (2020). Hair cortisol analyses in different mammal species: choosing the wrong assay may lead to erroneous results. *Conservation Physiology*, 8(1), coaa009. https://doi.org/10.1093/conphys/coaa009

Article 3: Azevedo A[1,2], Wauters J[1], Kirschbaum C[4], Serra R[5], Rivas A[6] & Jewgenow K[1] (2020). Sex steroids and glucocorticoid ratios in Iberian lynx hair. *Conservation Physiology*, 8(1), coaa075. https://doi.org/10.1093/conphys/coaa075

Article 4: Azevedo A[1,2], Bailey L[1], Bandeira V[3], Fonseca C[3], Wauters J[1] & Jewgenow K[1] (2021). Decreasing glucocorticoid levels towards the expansion front suggest ongoing expansion in a terrestrial mammal. *Conservation Physiology*, 9(1), coab050. https://doi.org/10.1093/conphys/coab050

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À minha querida esposa Rita, e ao meu querido filho Henrique.

Aos meus pais e irmãos.

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NON-INVASIVE MEASUREMENT OF LONG-TERM ADRENOCORTICAL ACTIVITY IN IBERIAN CARNIVORANS

Summary

The validation of enzyme immunoassays (EIAs) to measure hair glucocorticoids (hGCs) enables non-invasive assessments long term hypothalamic-pituitary-adrenal axis (HPA-axis) activity in captive and free-ranging wild animals, without capture or restraint. Despite the increasing application of these techniques, fundamental questions remain on how hGCs are incorporated into hair or relate to circulating levels, and EIAs using hair samples need to be rigorously validated for each species. This dissertation was designed to develop and validate EIAs to measure hGCs and assess long-term HPA-axis activity in two Iberian carnivorans: the endangered Iberian lynx (*Lynx pardinus*) and the thriving Egyptian mongoose (*Herpestes ichneumon*).

Article 1 details the development and validation of a cortisol EIA for hGC measurement in Egyptian mongoose. Analytical validation was performed and included a cross-validation with high-pressure liquid chromatography (HPLC) to assess EIA antibody specificity. Normative patterns of hGC variation for the species were established by assessing hGC variation with age, sex, season and sample storage time. The analytical and biological validity of the EIA was established by demonstrating (1) parallelism and acceptable coefficients of variation, (2) EIA-antibody binding to cortisol from hair extracts in HPLC immunograms, and (3) significant differences in hGC with age and sex in free-ranging Egyptian mongoose.

Article 2 highlights the challenge of overestimation of hGC measurements due to unintended EIA antibody cross-reactivity to non-target compounds present in hair extracts. We compared the performance of two different cortisol EIAs in identical hair extracts in the Iberian lynx and the Egyptian mongoose, and in four additional mammal species. The results of the EIAs were related to "gold-standard" liquid chromatography coupled with mass spectrometry (LC/MS-MS), and EIA antibody specificity was assessed using HPLC. The two EIAs overestimated hair cortisol to different extents. When compared with LC-MS/MS quantification, the best performing (cortisol-3CMO) EIA resulted in an up to three-fold overestimation of hair cortisol. Based on HPLC results, cross-reactivity with unknown compounds led to cortisol overestimation by the cortisol-21-HS EIA. In the case of the cortisol-3CMO EIA, major peaks of immunoreactivity were found in the elution positions of cortisol and cortisone, and EIA values correlated to the

sum of cortisol and cortisone LC-MS/MS measurements. The performance of each EIA was not consistent across species. Statistical analysis of hGC variation with age, sex, season and storage time, in a wild mongoose population, using the EIA with poorer performance led to erroneous results. This study demonstrates the need for careful methodological and biological validation to ensure that EIA measurements reflect hGC levels, and consequently HPA-axis activity.

Article 3 describes the validation of EIAs to measure steroids in Iberian lynx hair. EIAs for cortisol, cortisone and corticosterone (and progesterone and testosterone) were validated. EIA results were compared to “gold standard” LC-MS/MS, and EIA antibody specificity was assessed by HPLC. The variations of steroid measurements according to Iberian lynx age, sex and origin, as well as behaviour and management variables were analysed. Finally, we calculated cortisol-cortisone and cortisol-dehydroepiandrosterone (DHEA) ratios and assessed their relation with husbandry variables. The analytical and biological validity of the cortisol and cortisone EIAs was established by demonstrating (1) parallelism and acceptable coefficients of variation, (2) EIA-antibody binding to target hormones from hair extracts in HPLC immunograms, (3) correlation with LC-MS/MS measurements, and (4) significant differences in hGCs with age and sex in captive Iberian lynx. Corticosterone was not detected in Iberian lynx hair samples. Cortisol and cortisone exhibited opposing trends of variation with sex and origin, and in an individual animal after an escape-recapture event during a wildfire. Based on a limited number of samples, cortisol-cortisone and cortisol-DHEA ratios were apparently more sensitive to differences between captive-bred and wild-caught Iberian lynx held in captivity than the single hormones.

Article 4 illustrates the application of hGC measurement for the study of wild populations. The study assesses how spatial and environmental factors influence hGC measured with the previously validated EIA in an expanding population of Egyptian mongoose, using an information theoretic approach. A decrease in hGC was observed as the distance from the species’ historic range increased. Normal patterns of variation with age, sex, body condition and body size, had strong effects compared to spatial and environmental variables, illustrating the importance of accounting for their effect. Overall, the decrease of hair glucocorticoids toward the expansion front and the lack of an effect of human density and favourable habitat suggest the Egyptian mongoose expansion is unlikely to be limited by mechanisms related to- or mediated by the physiological

stress response. This study was the first to measure glucocorticoid (GC) variation in a mammalian range expansion, and to use a matrix representing a long-term integrated measure of GCs. It illustrates how hGC measurement can contribute to the understanding of how mammals are influenced by environmental factors by identifying variables that influence long-term adrenocortical activity, potentially facilitating the species' expansion.

Keywords: hair; glucocorticoids; stress

Resumo

A validação de imunoensaios enzimáticos (EIAs) para a medição de glucocorticóides no pêlo (hGCs) viabiliza estudos não-invasivos da actividade do eixo hipotálamo-pituitária-adrenal (HPA) em animais selvagens em mantidos cativeiro ou em liberdade, sem recurso a captura ou contenção. Apesar da aplicação crescente destas técnicas, persistem questões fundamentais quanto à incorporação dos glucocorticóides (GCs) nos pêlos e à sua relação com níveis séricos. Além disso, os EIAs carecem de validação rigorosa para cada espécie. Esta dissertação foi desenvolvida no sentido de validar EIAs que permitam medir hGC e a actividade do eixo HPA em dois carnívoros Ibéricos: o lince-Ibérico (*Lynx pardinus*), uma espécie ameaçada, e o sacarrabos (*Herpestes ichneumon*), uma espécie em expansão.

O **Artigo 1** detalha o desenvolvimento e a validação de um EIA de cortisol para a medição de hGC em sacarrabos. O EIA foi validado analiticamente, incluindo a validação cruzada com cromatografia líquida de alta pressão (HPLC) para avaliar a especificidade dos anticorpos do imunoensaio. Os padrões normais de variação de hGC para a espécie foram estabelecidos através da caracterização da sua variação com a idade, o sexo, a sazonalidade e o tempo de armazenamento das amostras. A validade analítica e biológica do EIA foi demonstrada através de (1) paralelismo e coeficientes de variação aceitáveis, (2) a ligação do anticorpo do EIA ao cortisol em imunogramas obtidos através de HPLC, e (3) diferenças significativas nas concentrações de hGC com a idade e o sexo em sacarrabos selvagens.

O **Artigo 2** aborda a sobrestimação de medições de hGC devido à reactividade cruzada dos anticorpos dos EIAs com vários compostos presentes nos extractos de pêlo. O estudo compara o desempenho de dois EIAs diferentes para cortisol em extractos idênticos de pêlo de sacarrabos, lince-Ibérico e quatro outras espécies de mamíferos. Os resultados das medições por EIA foram comparados com os obtidos por cromatografia líquida acoplada a espectrometria de massa (LC-MS/MS) e a especificidade dos anticorpos dos EIA foi avaliada através de HPLC. Os dois EIAs sobrestimaram o cortisol nos extractos de pêlo em diferentes medidas. Em comparação com os resultados de LC-MS/MS, mesmo o EIA com melhor desempenho (cortisol-3CMO) resultou numa sobrestimação de até três vezes. Os imunogramas de HPLC revelaram que a sobrestimação de cortisol se

deveu a reactividade cruzada com compostos desconhecidos no EIA cortisol-21-HS, e com cortisona no EIA cortisol-3CMO. Neste último, os valores medidos por EIA estavam correlacionados com a soma dos valores de cortisol e cortisona medidos por LC-MS/MS. O desempenho dos EIA variou de acordo com as espécies. Na população de sacarrabos selvagens, a análise da variação de hGC com a idade, sexo, sazonalidade e tempo de armazenamento, usando o EIA com pior desempenho levou a resultados erróneos. Este estudo demonstra a necessidade da validação metodológica e biológica para assegurar que as medições de EIA reflectem os níveis de hGC no pêlo, e consequentemente a actividade do eixo HPA.

O **Artigo 3** descreve a validação de EIAs para a medição de hormonas esteróides em pêlo de lince-Ibérico. Neste estudo são submetidos a validação EIAs para cortisol, cortisona e corticosterona (bem como progesterona e testosterona). Os resultados dos EIAs foram comparados a medições por LC-MS/MS dos mesmos extractos, e a especificidade dos anticorpos dos imunoensaios foi verificada por HPLC. Foram caracterizadas as variações das hormonas de acordo com a idade, sexo e origem dos linces, e com variáveis de maneio e de comportamento. Finalmente, calcularam-se os rácios cortisol-cortisona e cortisol-dehydroepiandrosterona (DHEA) e avaliou-se a sua relação com variáveis de maneio. A validação analítica e biológica dos EIAs de cortisol e cortisona foi conseguida demonstrando (1) o paralelismo e coeficientes de variação aceitáveis, (2) a ligação dos anticorpos dos EIA às hormonas alvo nos extractos de pêlo através de imunogramas de HPLC, (3) a correlação das medições por EIA com as medições por LC-MS/MS, e (4) diferenças significativas das concentrações de hormonas no pêlo de acordo com idade e sexo numa população de linces-Ibérico mantido em cativeiro. Não foi detectada corticosterona no pêlo de lince-Ibérico. O cortisol e cortisona exibiram padrões de variação opostos com o sexo e a origem dos linces, e num espécime de lince após um evento de fuga e recaptura durante um incêndio florestal que atingiu o centro onde estava alojado. Embora com base num número limitado de animais, os rácios de cortisol-cortisona e cortisol-DHEA foram mais sensíveis que as hormonas individuais na detecção de diferenças entre linces-Ibéricos nascidos em cativeiro ou no estado selvagem.

O **Artigo 4** ilustra a aplicação práctica da medição de hGCs ao estudo de populações selvagens de animais. O estudo aplica uma abordagem de teoria da informação para investigar a influência de factores espaciais e ambientais sobre

os hGCs numa população de sacarrabos em expansão geográfica, usando um EIA previamente validado. A análise revelou uma diminuição de hGC em direcção à frente de expansão, isto é, à medida que a distância da área de distribuição histórica da espécie aumenta. As variáveis idade, sexo, condição corporal e tamanho corporal demonstraram efeitos mais fortes que variáveis espaciais e ambientais, o que ilustra a importância de considerar o efeito das variações normais de hGC na análise. No geral, o decréscimo de hGC em direcção à frente de expansão e a ausência de um efeito da densidade populacional humana e da área de habitat favorável sugerem que a expansão do sacarrabos não está a ser limitada por mecanismos relacionados ou mediados pela actividade do eixo HPA. Este estudo é o primeiro a medir variações de GCs associados à expansão geográfica de um mamífero, e a usar amostras que representam valores de GCs por um período alargado para o efeito. Ao identificar as variáveis que influenciam a actividade adrenocortical a longo prazo e que poderão ter facilitado a expansão do sacarrabos, os resultados deste trabalho ilustram o potencial da medição de hGCs para ajudar a compreender a forma como populações ou espécies de mamíferos selvagens são influenciadas por factores ambientais.

Keywords: pêlo; glucocorticóides; *stress*

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1. Introduction

Recent assessments estimate that one million species are currently threatened with extinction (Díaz *et al.*, 2020). Even where extinction is not an immediate threat, populations of wild species are experiencing severe declines and extirpations, in what has been termed "biological annihilation" (Ceballos *et al.*, 2017). The current extinction rate exceeds that of historical mass-extinction events (Pimm *et al.*, 2014; Ceballos *et al.*, 2015) and threatens humanity with the loss of irreplaceable ecosystem services that underpin life as we know it (Díaz *et al.*, 2020). Thus, humanity faces the unprecedented challenge of reversing the trend of biodiversity loss while satisfying the needs of a growing human population (Leclère *et al.*, 2020). As this struggle slowly progresses at the global policy-making level, many local efforts have successfully prevented or reversed declines and extirpations by protecting habitats and restoring populations of threatened species, providing valuable lessons on how to reverse the current trend (Johnson *et al.*, 2017).

The two species targeted in this work are both extreme examples of the impacts of anthropogenic environmental change on wild species. At the end of the twentieth century, the Iberian lynx (*Lynx pardinus*), a top-order predator endemic to the Iberian Peninsula, was the most endangered feline species in the world (Nowell *et al.*, 1996) with approximately 100 individuals remaining in the wild (Guzmán *et al.*, 2004). Successful conservation efforts have increased population numbers, restored this felid to many areas of its historical range (Simón *et al.*, 2012; Rodríguez and Calzada, 2015) and prevented further loss of genetic variability (Kleinman-Ruiz *et al.*, 2019). Nevertheless, the species remains threatened by the lowest genome-wide diversity reported so far for any species (Abascal *et al.*, 2016) and faces an uncertain future in the face of changing climate (Fordham *et al.*, 2013). At the same time, the Egyptian mongoose (*Herpestes ichneumon*), a small carnivore sympatric to the Iberian lynx, had started expanding its distribution throughout the Iberian Peninsula. Its distribution had been confined to the South of the Tagus River until the mid-1990's, but since then it has experienced a continuous and rapid northward range expansion (Barros *et al.*, 2015, 2016). The opposing trends observed in

these two species illustrate the striking differences in how each species may be impacted by anthropogenic environmental change (Clavel *et al.*, 2011).

Variations in each animal's response to environmental disturbance are mediated by the physiological processes that influence its ability to cope with stressors, survive and produce offspring. As mediators of these physiological processes, hormones have been targeted as indicators, placing research in endocrinology at the root of many conservation successes. Studies focusing on endocrine indicators have made essential contributions for the successful establishment of captive breeding programmes, by providing critical information on reproductive and stress physiology (Jewgenow and Songsasen, 2014; Kersey and Dehnhard, 2014). The contributes of reproductive science to conservation programmes are fundamental and widespread, but beyond the scope of this work (reviewed in Comizzoli *et al.*, 2019). Examples of studies using stress physiology to inform conservation programmes are becoming increasingly abundant. For example, in the black-footed ferret (*Mustela nigripes*), glucocorticoid analyses have been used to understand the effects of environmental enrichment (Poessel *et al.*, 2011) and nutrition (Santymire *et al.*, 2020) on the stress physiology of the population in the captive breeding programme. In other species, glucocorticoids measurements have been used to understand stress during reintroduction (e.g. Hartup *et al.*, 2005; Zidon *et al.*, 2009; Jachowski *et al.*, 2013; Batson *et al.*, 2017), in response to disturbance (Creel *et al.*, 2009; Dantzer *et al.*, 2014; Bryan *et al.*, 2015; Vilela *et al.*, 2020) or with naturally occurring ecological processes (Creel *et al.*, 2009; Sheriff *et al.*, 2011b; Bryan *et al.*, 2014; Dulude-de Broin *et al.*, 2020). On a macro-ecological scale, variations in glucocorticoid levels could offer a mechanistic understanding of the effects of environmental change on organisms (Wikelski and Cooke, 2006; Chown and Gaston, 2008; Seebacher and Franklin, 2012; McCormick and Romero, 2017). Despite the challenges to obtaining comparable data of several species, efforts are underway to compile the results of steroid hormone measurements in different populations and species and allow meta-analyses (Vitousek *et al.*, 2018a).

1.1. Glucocorticoids and the stress response

Among the physiological data frequently collected from wild animals are the measures that provide information on the physiological (stress) response of organisms to the challenges that ever-changing environments pose to their internal stability (homeostasis). The stress response is essential to enable animals to cope with changes in the environment. It comprises a suite of coordinated molecular, behavioural and physiological mechanisms, including the neuroendocrine hypothalamic-pituitary-adrenal axis (HPA-axis) that culminates in glucocorticoid release (Moberg, 2000; Charmandari *et al.*, 2005; Reeder and Kramer, 2005). When an animal perceives a potentially disrupting stimulus, two physiological responses are triggered. Within seconds, the sympathetic adrenomedullary system induces the release of epinephrine and norepinephrine into circulation, initiating the "*fight or flight response*" (Reeder and Kramer, 2005; Chrousos, 2009). Simultaneously, corticotropin-releasing hormone (CRH) is secreted by the hypothalamus into the hypophyseal portal system, inducing the release of adrenocorticotropic hormone (ACTH) by the pituitary gland into the blood stream. As a result of the ACTH increase, the adrenal cortex increases the secretion of glucocorticoids, prompting an array of effects on virtually all peripheral tissues in order to help the animal return to a stable (homeostatic) state (Sapolsky *et al.*, 2000; Charmandari *et al.*, 2005). The effects of glucocorticoids are essential but not limited to the stress response. Basal levels of circulating glucocorticoids are necessary for normal organism function (Sapolsky *et al.*, 2000) and vary to account for specific metabolic needs in different life-history stages (Romero, 2002; McEwen and Wingfield, 2003). When faced with environmental change, basal levels of glucocorticoids prime the acute behavioural and physiological responses to stressors, while elevations in glucocorticoid levels caused by HPA-axis activation enhance the acute response and simultaneously prevent a potentially damaging overshoot and promote the restoration of metabolic reserves and normal behaviour, in order to prepare the organism for the next challenge (Sapolsky *et al.*, 2000). The temporary elevation in circulating glucocorticoids in response to acute stressors such as a predatory or aggressive interaction helps animals cope with emergencies presented by a dynamic environment (Wingfield *et al.*, 1998) and constitutes an adaptive mechanism that is fine-tuned through genetic and epigenetic processes and subject to environmental selection (Welberg and Seckl, 2001). However, when chronic,

repeated or uncontrollable stressors overwhelm animals' ability to cope, the HPA-axis response can become maladaptive or pathological (Sapolsky *et al.*, 2000; McEwen and Wingfield, 2003; Romero *et al.*, 2009). While catecholamines remain elevated in circulation for only short periods, glucocorticoid peak levels occur hours after ACTH release, and their effects can last for days (Sapolsky *et al.*, 2000). Therefore, glucocorticoid measurements have been the focus of research seeking to understand the response to stress in individuals, populations or species (Wikelski and Cooke, 2006; Dantzer *et al.*, 2014), and at a macro-ecological scale (Chown and Gaston, 2008; Jessop *et al.*, 2013).

1.2. Non-invasive analysis of glucocorticoids

Cortisol and corticosterone are the main glucocorticoids secreted by the HPA-axis (or the hypothalamic-pituitary-interrenal axis in fish, amphibians and reptiles) in vertebrates, and have therefore been extensively used as indicators of HPA-axis activity in wildlife (Sheriff *et al.*, 2011a). Cholesterol is the precursor of all steroid hormones from which cortisol, the primary endogenous glucocorticoid in most mammals, is synthesized after several steps of enzymatic conversion in the *zona fasciculata* of the adrenal cortex (figure 1). Active cortisol exerts its effects through binding with glucocorticoid or mineralocorticoid receptors in cells, and can be reversibly inactivated by conversion to cortisone by the 2 isoforms of 11β-hydroxysteroid-dehydrogenase, 11β-HSD2 and 11β-HSD1 (Sapolsky *et al.*, 2000).

The methods most commonly used to quantify glucocorticoids and their metabolites in wildlife are enzyme immunoassay (EIA) and radioimmunoassay (RIA) (Sheriff *et al.*, 2011a), which have been increasingly complemented by the use of other methods like liquid (or gas) chromatography coupled with mass spectrometry (LC/MS-MS). Glucocorticoid measurements in plasma have provided important information on HPA-axis reactivity, the acute response to stress and how it varies seasonally and with life-history stages (e.g. Romero *et al.*, 2000, 2008; Romero, 2002). However, numerous point measures of plasma, urine or salivary cortisol would be necessary to provide an indication of chronic stress, making it impractical. Rapid changes in circulating cortisol levels in response to acute stressors (Sapolsky *et al.*, 2000) and due to the circadian and ultradian rhythms of cortisol secretion (Lefcourt *et al.*, 1993; Leliavski *et al.*, 2015; Palme, 2019) further confound the use of plasma glucocorticoid measurements in the assessment of chronic HPA-axis activation. Additionally, the collection of blood

samples is an invasive procedure that requires capture and restraint, and therefore may not be feasible in endangered species or for the study of free-ranging wild animals. This has led to the development of methods that are non-invasive and provide integrated measures of circulating glucocorticoids such as measurement of faecal glucocorticoid metabolites and hair glucocorticoids. Because they reflect cumulative glucocorticoid levels for the previous days (faeces) or weeks (hair), these measures are expected to more efficiently detect pathological or maladaptive elevations in adrenocortical activity resulting from chronic or repeated environmental challenges that overwhelm animals' ability to cope.

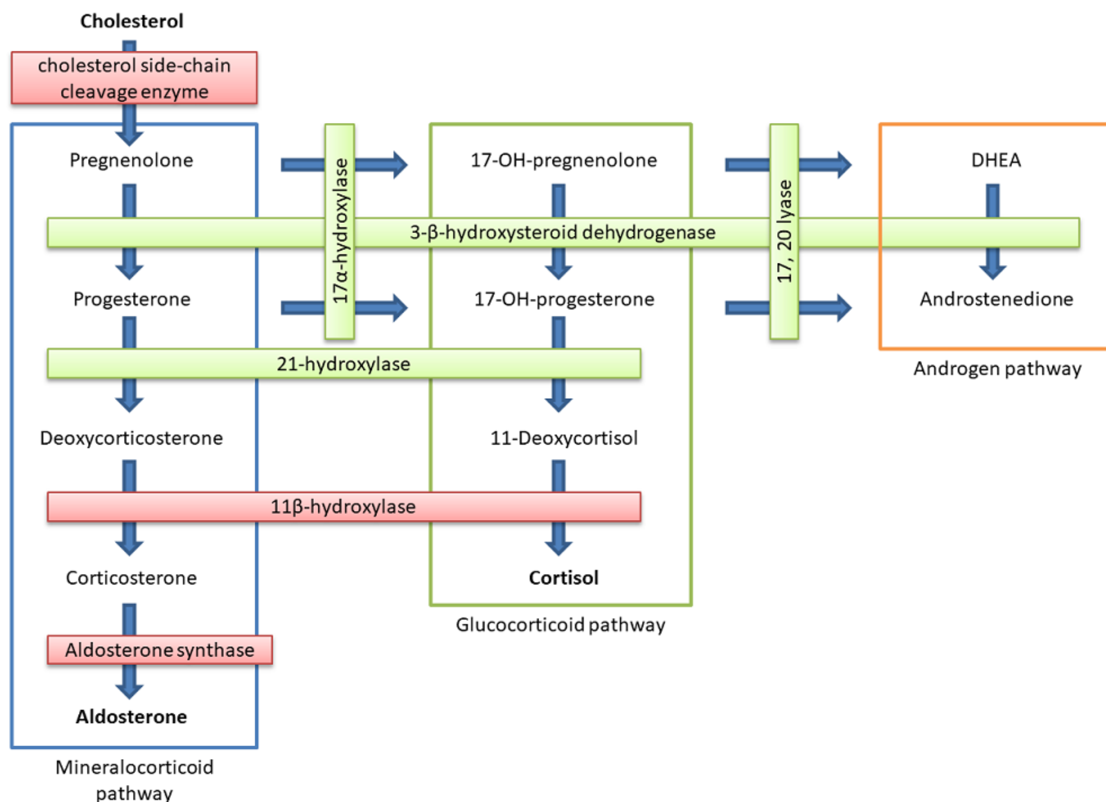

Figure 1. Simplified representation of steroidogenic pathways. Cholesterol is the precursor of biologically active steroids synthetized in the three different zones of the adrenal cortex. Mineralocorticoid synthesis takes place in the *zona glomerulosa*, glucocorticoid synthesis occurs in the *zona fasciculata* and androgens are synthesized in the *zona reticularis*. Enzymes in green boxes are located in the smooth endoplasmic reticulum; enzymes in red boxes are located in mitochondria. Adapted from Häggström *et al.* (2014).

1.3. Validation of non-invasive glucocorticoid analyses

Methods for measuring glucocorticoids require analytical, physiological and biological validation (Touma and Palme, 2005). Caution regarding the need for rigorous validation in order to obtain meaningful results has been advised for measurement of faecal glucocorticoid metabolites (Touma and Palme, 2005; Palme, 2019). In order to develop methods to non-invasively measure glucocorticoids in a new species, analytical validation must be performed to demonstrate that the EIA has acceptable specificity, accuracy and precision (Touma and Palme, 2005; Sheriff *et al.*, 2011a; Kersey and Dehnhard, 2014; Palme, 2019). This requires consideration of how samples are collected and stored, validation of extraction methods and of the assay itself. Physiological validation is achieved by demonstrating that the measures obtained from the EIA reflect the underlying physiological process, or in other words, that the glucocorticoid measurements reflect variations in circulating glucocorticoid levels. Physiological validation is typically achieved by combining several methods, namely measuring glucocorticoid increases in response to ACTH injections (del Rosario *et al.*, 2011; Ludwig *et al.*, 2013; Terwissen *et al.*, 2013; Mastromonaco *et al.*, 2014), by correlation with other previously validated indicators of stress (Turner *et al.*, 2002; Davenport *et al.*, 2006) and by using radiolabelled hormone injections to characterize their metabolism and excretion pathways (Dantzer *et al.*, 2010; Benhaiem *et al.*, 2012; Keckeis *et al.*, 2012; Ludwig *et al.*, 2013; Kapoor *et al.*, 2018). Finally, biological validation is required in order to ascertain whether the changes in glucocorticoid levels reflect the effect of stressors on the organism, and consequently enable meaningful interpretations of the results. An example of biological validation can be found in Davenport *et al.* (2006), where cortisol levels were assessed before and after relocation of non-human primates. However, several factors can affect the biological validity of the results, including confounding effects of experimental design such as the effect of restraint and handling, or normative patterns of variation in glucocorticoids according to sex (Bechshøft *et al.*, 2011; Cattet *et al.*, 2014), season (Martin and Reale, 2008; Cattet *et al.*, 2014) or life-history stages (Möstl and Palme, 2002; del Rosario *et al.*, 2011; Fanson *et al.*, 2012; Dettmer *et al.*, 2014). Only after all these differences are accounted for and biological validity established, should glucocorticoid measurements be used to assess the effect of stressors on individuals and populations.

1.4. Aims of this thesis

A retrospective measure of HPA-axis activity over a period of several weeks would provide a physiological indicator of chronic stress. In wildlife, such data could provide crucial and timely information on the impacts of environmental change and the success of conservation efforts. The aim of this work is to develop and validate non-invasive methods to measure HPA-axis activity in the Iberian lynx and the Egyptian mongoose, using hair glucocorticoid analyses. In order to obtain meaningful hair glucocorticoid measurements, analytical and physiological validation of EIAs was performed in both species. Next, normal patterns of hair glucocorticoid variation with sex, age and season were characterized, in order to finally enable the assessment of hair glucocorticoid variation with candidate stressors. Throughout the process, we aimed to address and discuss major knowledge gaps regarding hair glucocorticoid analyses. For this purpose, several methods were used for cross-validation (LC/MS-MS and HPLC) and several steroids were measured in hair samples. Finally, the validated methods were applied to a wild population of Egyptian mongoose that is expanding its range, in order to provide an example of the method’s potential to increase our understanding of how species respond to changing environmental conditions.

2. Article 1 – hair glucocorticoid analysis in the Egyptian Mongoose

Azevedo A, Bailey L, Bandeira V, Dehnhard M, Fonseca C, De Sousa L & Jewgenow K (2019). Age, sex and storage time influence hair cortisol levels in a wild mammal population. *PLOS OnE*, 14(8), e0221124.
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The aim of this work was to validate a cortisol EIA as an indicator of HPA-axis activity in the Egyptian mongoose. The first step to achieve this purpose was the analytical validation of the EIA. Next, HPLC was used to determine whether the assay was targeting cortisol, by comparing the elution positions of the EIA with the elution positions determined for authentic steroids. In order to contribute to biological validation, we determined the normative patterns of variation of EIA cortisol measurements in hair samples from wild mongoose covering the species entire distribution in Portugal. We assessed the effect of age, sex, season and sample storage time, while accounting for spatial and temporal variation, using linear mixed effects models. Finally, we assessed the effect of female reproductive state by fitting a model with female data only.

Author contributions:

AA: conception and design, laboratory analysis and data acquisition, analysis and interpretation, writing, review and editing; LB: analysis and interpretation, writing, review and editing; VB: data acquisition, review and editing; MD: design, laboratory analysis, analysis and interpretation, review and editing, supervision; CF: review and editing; LS: supervision, review and editing; KJ: conception and design, analysis and interpretation, review and editing, supervision.

Results of the study:

1. Cortisol levels in Egyptian mongoose hair were determined with an EIA using a polyclonal antibody against cortisol-3-CMO-BSA and cortisol-3-CMO-peroxidase as label.

2. Based on HPLC analysis, cortisol was the major glucocorticoid detected by the assay in Egyptian mongoose hair, corresponding to 40% of the total immunoreactivity. Three minor peaks of immunoreactivity were detected,

one of which corresponded to authentic cortisone's elution position, while the other two did not co-elute with any of the steroid standards.

3. In wild Egyptian mongoose, hair cortisol was higher in juveniles between two-and-a-half and five-and-a-half months compared to all other age classes. Hair cortisol was also higher in males than in females, and decreased with sample storage time.

4. There was no effect of reproductive state on hair cortisol measurements in wild Egyptian mongoose females.

5. Baseline variations of hair cortisol in wild Egyptian mongoose in Portugal were established, thus enabling its use as an indicator of chronically elevated HPA-axis activity.

PLOS ONE

RESEARCH ARTICLE

Age, sex and storage time influence hair cortisol levels in a wild mammal population

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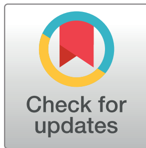

Abstract

The measurement of hair cortisol is increasingly used to understand the effect of natural and anthropogenic stressors on wild animals, but it is potentially confounded by individual, seasonal and sex-dependant variations in baseline cortisol secretion. This study validated an enzyme-linked immunoassay for hair cortisol measurement and characterized its baseline variation in a wild population of Egyptian mongoose. The analysis encompassed individuals of both sexes and all ages, across a range of geographic, environmental and seasonal conditions that the species experiences in Portugal allowing us to account for spatial, temporal and biological factors that contribute to hair cortisol variation. Our results showed that age, sex and storage time had an effect on hair cortisol, but season did not. Hair cortisol was higher in early stage juveniles compared to other age cohorts, in males when compared to females, and decreased with longer storage time. By identifying the factors that influence baseline hair cortisol in this wild population, we establish the basis for its application as an indicator of the effect of natural and anthropogenic stressors.

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Introduction

In the Anthropocene, wild animal populations are faced with a broad range of environmental stressors from a mix of both anthropogenic and natural sources. Although consequences such as species declines and ecosystem imbalances are often measurable, causal mechanisms underlying conservation problems are more difficult to establish and require an understanding of the physiological responses of animals to environmental change [1]. This has led to the emergence of the field of conservation physiology and to a growing interest in understanding how environmental change affects the physiology of wild animal populations, with important implications for wildlife management and conservation. One way to study the effects of environmental stressors is through the measurement of substances that mediate the physiological stress response. In mammals, cortisol is a key mediator of the hypothalamic-pituitary-adrenal stress response. While acute changes in its circulating levels are necessary to maintain homeostasis in face of a dynamic environment, chronic elevations may impact immunity, behaviour and reproduction [2–4]. In controlled settings, cortisol is often measured in blood plasma and

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serum, but this only provides a snapshot of current cortisol levels, which can be influenced by the stress of the sampling procedure [4,5]. In addition strong ultradian rhythms with periods around 120 min or less may result in extreme fluctuations within individuals over time [6,7]. Consequently, non-invasive approaches have been developed using alternative sample matrices such as urine, faeces or saliva to measure glucocorticoid metabolites that mirror adrenocortical (stress) status of the preceding hours or days [4,5]. More recently, the measurement of cortisol in hair has been used to provide an integrated value of circulating cortisol over a period of several weeks [8–14]. The exact sources of cortisol in found hair are not fully understood and include both incorporation of circulating cortisol from blood vessels and tissues surrounding follicular cells in growing hair [15] and production by a local HPA-axis analogue [16–18]. Nevertheless, evidence that hair cortisol reflects changes in circulating levels in cases of hypoadrenocorticism [19], hyperadrenocorticism [20], consecutive ACTH injections [11,14] and major life stressors [10,21] indicates that circulating cortisol levels are reflected in hair. Finally, this approach offers great potential as an indicator of chronic stress because it is unaffected by sampling procedures, ultradian rhythms or globulin-bound cortisol [22].

Before hair cortisol can be broadly used as an indicator of stress, it is first necessary to investigate how much of observed hair cortisol variations can be attributed to stress and disturbance in relation to baseline and individual variation. Each individual's baseline adrenocortical activity and HPA-axis reactivity is influenced by genetic inheritance [23,24], maternal and epigenetic effects [25–27], biotic environmental factors like infection or predation risk [28,29] and abiotic factors like weather and climate [30,31]. Additionally, glucocorticoid levels may vary with sex [32], developmental stage [33,34] and age [11,35]. Although often not evident in studies using captive animals, seasonal variations in glucocorticoids levels have been demonstrated in wild species [36,37]. Highest levels of glucocorticoids are mostly related with the breeding season in birds and reptiles but in mammals this trend is less clear, with some species showing higher glucocorticoid levels during breeding season and others showing post-breeding increases [37,38]. In addition, several methodological issues need to be considered, since storage time, storage conditions, anatomical location [9,12,14] and type [12] and colour of the hair sample [11] have been shown to influence the amount of cortisol retrieved. Failure to account for these sources of variation in glucocorticoid levels may confound their interpretation and lead to a misestimation of the effects of disturbance.

The Egyptian mongoose (*Herpestes ichneumon*) is a medium-sized carnivore that colonized the Iberian Peninsula in the Pleistocene [39] and has experienced a recent expansion in its distribution due to several biotic and abiotic factors [40–42]. The species has a polygynic mating system, with different levels of investment by males and females in reproduction [43,44], leading to physiological and behavioural differences between sexes that might also be reflected in hair cortisol levels, especially during the breeding season. The widespread distribution and ongoing expansion of *H. ichneumon*, covering almost all types of habitat in Portugal [42], coupled with the availability of captured specimens [45,46], makes it a useful model for the study of hair cortisol as an indicator of stress.

In this study we determined hair cortisol in samples from wild caught Egyptian mongoose specimens with known sex, age, location and date of capture. We hypothesize that age, sex, and season will have an effect on hair cortisol variation, with effects of age and season likely to vary between sexes. We also considered how female reproductive state (non-breeding, lactating, pregnant) might affect hair cortisol measurements, with higher hair cortisol levels expected in breeding individuals. Through this work we aim to describe baseline variation in hair cortisol levels, which will contribute to a better understanding of the potential for hair cortisol to be used as an indicator of stress in wild animal populations. In addition HPLC analyses was carried out to investigate the profile of glucocorticoids that cross-react with our cortisol

antibody as data from Keckeis et al. [47] demonstrated large amounts of immunoreactivity not coinciding with cortisol suggesting a local production of glucocorticoids in hair follicles.

Methods

Sample collection

We obtained hair samples from carcasses of wild Egyptian mongoose that were obtained from hunting activities in seven provinces of mainland Portugal between January 2008 and December 2014, in compliance with legal requirements and with permits from the competent authorities in Portugal, the ICNF–Instituto da Conservação da Natureza e das Florestas [45]. Following death, specimens had remained frozen at -20˚C until the date of sample collection. After thawing, hair was clipped with scissors as close to the skin as possible from a standard area between the shoulders to account for variation in cortisol levels due to anatomical location. Samples were stored in paper envelopes in a dark dry location until the date of extraction. Storage time was defined as the total number of days between the date of capture of the mongoose and the date of cortisol extraction from hair.

Age was obtained based on dental development, with each mongoose classified as an adult (over one year of age), sub-adult (between nine and twelve months), type II juvenile (between five-and-a-half and nine months) and type I juvenile (between two-and-a-half and five-and-a-half months of age), following methods of Bandeira et al. [45].

Each specimen was assigned as male or female based on the presence of testicles or ovaries, and the reproductive state of females was noted as pregnant (foetuses identified in the uterus), lactating (milk present in mammary glands) or non-breeding.

Sample season was assigned based on the date of capture. In confirmation with other ongoing studies, animals captured from October to December were included in the autumn class, from January to March in the winter class, from April to June in the spring class and from July to September in the summer class.

A total of 294 Egyptian mongoose hair samples were collected for this study. Age cohort could not be determined for 50 specimens, which were excluded from the statistical analysis. Of the 244 mongooses with known age class, 114 were males and 130 were females (12 of which were pregnant and 7 lactating). In terms of age cohorts, 147 were adults, 27 were sub adults, 40 were type II juveniles, and 30 were type I juveniles. The number of specimens in each of the 7 provinces varied between 2 (Estremadura) and 134 (Baixo Alentejo) and sample storage time varied from 863 to 2,266 days.

Sample preparation

All chemical reagents were purchased from Sigma–Aldrich (Taufkirchen, Germany) unless stated otherwise and were of the highest purity available.

Approximately 20 mg of full-length guard hairs were manually separated from undercoat hairs and placed in Eppendorf tubes. Guard hairs and undercoat are clearly distinguishable in Egyptian mongoose hair samples. In order to avoid variation due to the incorporation of different proportions of each hair type, we decided to use only one type of hair. Guard hairs were chosen because they are more likely to be retrieved from hair traps in future applications than undercoat, and allow comparison with similar studies in other species [e.g. 12].

To remove surface contamination, 2 mL of 90% methanol was added to the hair sample and vortexed for 5–10 seconds. Following settling, methanol was discarded and the wash step was repeated. After the two washes, samples were dried for one hour at 70˚C. Next, 10 mg (8.29–12.12 mg) of washed hair were removed, ceramic beads (six 2.8 mm beads and 600 ± 10 mg of 1.4 mm beads) were added, and hairs were ground to a fine powder in a Precellys24

tissue homogenizer (Bertin Technologies, France). For cortisol extraction, 400 μL of 90% methanol were added to 10 mg of hair powder in separate tubes and shaken at room temperature for 30 minutes using a universal shaker (SM-30, Edmund Buhler GmbH, Hechingen, Germany). Samples were centrifuged (3 min, 1000G), the supernatant was collected and transferred to a new tube, diluted 1:2 with water and frozen until the day of cortisol measurement.

High-performance liquid chromatography (HPLC)

To confirm cortisol as the major hair glucocorticoid in the Mongoose, hairs extracts were used for HPLC analysis. To obtain an appropriate cortisol concentration for HPLC analysis 150 μL of 294 hair extracts (in 40% methanol) corresponding to 550 mg hair were pooled and purified on a C18 column (ecf, Chromabond, Macherey–Nagel, Düren, Germany). For this purpose the pooled extract was diluted to 10% methanol with water. The C18 column was equilibrated with 2 mL 100% methanol followed by 2 mL of 20 mM Tris buffer (pH 8.5) containing 10% methanol. After applying the pooled hair extract on the C18 column it was washed twice with 2 mL of 20 mM Tris buffer (pH 8.5) containing 10% methanol. The purified sample was eluted with 3 mL of 100% methanol, evaporated in a sample concentrator (Dri Block DB3, Techne, Staffordshire, UK) under a constant nitrogen flow and finally resuspended in 120 μL of 100% methanol and 180 μL of water resulting in 300 μL purified extract in 40% methanol.

HPLC analysis was performed as described before [48]. In brief, 150 μL of the purified extract were separated on a reversed-phase Ultrasep ES⁄RP– 18⁄6 μm HPLC column (4x250 mm; Sepserv, Berlin, Germany). Glucocorticoids were separated using a methanol + water mixture with the following gradient: 60% methanol over 5 min, 60–90% methanol over 10 min, 90% methanol for another 10 min. The flow rate was 1 mL/min. Fractions of 0.33 mL were collected at 20 sec intervals over a period of 25 min and diluted with one volume of water. All fractions were lyophilized and resuspended in 200μL 40% methanol. The elution positions of authentic cortisol, hydrocortisone, corticosterone, cortisone, testosterone, dihydrotestosterone, 11β-hydroxyetiocholanolone and progesterone had been previously determined in separate HPLC runs.

Cortisol measurement

Cortisol was quantified by an enzyme immunoassay (EIA) using a polyclonal antibody (rabbit) against cortisol-3-CMO-BSA and cortisol-3-CMO-peroxidase as label. The antibody cross-reactivities to different steroids were as follows: 4-pregnen-11α,17,21-triol-3,20-dione (cortisol), 100%; 5α-pregnan-3β,11β,17,21-tetrol-20-one (3β,5α-tetrahydrocortisol), 8.4%; 4-pregnen-11β,21-diol-3,20-dione (corticosterone), 6.3%; 5α-pregnan-11β,17,21-triol-3,20-dione (5α-dihydrocortisol), 3.2%; and <0.1% for 4-pregnen-21-ol-3,20-dione (desoxycorticosterone), 5β-pregnan-11β,17,21-triol-3,20-dione (5β-dihydrocortisol), 5α-pregnan-3α,11β,17,21-tetrol-20-one (5α-tetrahydrocortisol), 5β-pregnan-3α,11β,17,21-tetrol-20-one (5β-tetrahydrocortisol), 4-pregnen-3,20-dione (progesterone), 5α-pregnan-3,20-dione, 5α-pregnan-3β-ol-20-one, dexamethasone, estradiol, and testosterone [49]. Duplicates of 10 μl hair extract or cortisol standards prepared in 40% methanol ranging from 0.2 to 100 pg/20 μl were then simultaneously pipetted into respective wells along with 100 μl cortisol-HRP conjugate in assay buffer (50 mM Na_2HPO_4/Na_2HPO_4, 0.15 M NaCl, 0.1% BSA, pH 7.4) with the aid of a diluter dispenser. Then, 100 μl of cortisol-specific antibody in a final dilution of 1:400.000 were added. After overnight incubation at 4˚C, the assay was terminated following our standard protocol, described in Finkenwirth et al. [50]. Assay validation was performed by demonstrating parallelism of serially diluted hair extracts to the cortisol standard curve.

Statistical methods

Effects of age, sex, season, and storage time. Statistical analyses were conducted in R (v3.5.1) using linear mixed effects models with a Gaussian error distribution from the package lme4 [51]. We considered the effect of mongoose age and sex, the season in which samples were collected and the storage time (days) on hair cortisol concentration (pg/mg). We also considered the possibility for interactions between sex and both season and age. Our model included a random intercept term for the province in which samples were collected (7 levels), and both the year and month in which samples were collected (12 months within each of 5 years) to account for potential spatial and temporal variation in hair cortisol concentration. Intercepts of random effects were assumed to follow a Gaussian distribution.

Variance inflation factors were used to test for multi-collinearity between variables. No evidence of multi-collinearity was detected using a variance inflation factor cut-off of 3. We identified two outlier values that were more than four times larger than median hair cortisol and almost twice as high as the next highest measurement (Cook's distance of 0.38 and 0.63). Four-fold increases in hair cortisol have previously been observed in response to repeated ACTH-challenge in dairy cattle and eastern chipmunks (11,13), and so these values may be biologically plausible in situations of chronic and severe stress. We conducted all analyses with and without these outliers included. Results with outliers removed are presented in the main text while results with and without outliers included are provided in tables.

Effect of female reproductive state. Female reproductive state (non-breeding, pregnant, lactating) may also be an important factor affecting hair cortisol concentration. Therefore, we also fitted a general linear mixed effects model using female data only (N = 130). We considered the effect of female age and reproductive state, the season in which samples were collected and the storage time of samples (days) on hair cortisol concentration (pg/mg). The random effects structure of the model was the same as above.

Significance testing. Variable significance ($\alpha = 0.05$) was determined using parametric bootstrapped likelihood ratio tests with 5,000 iterations using the package pbkrtest [52]. The parametric bootstrapping approach conducts multiple simulations of a likelihood ratio test and returns the fraction of simulated likelihood ratio test values that are larger or equal to the observed likelihood ratio test statistic taken from the true data (i.e. a significant result is one where the observed likelihood ratio test value is lower than 5% of simulated likelihood ratio test values). A model with each fixed effect parameter removed was compared to the global model containing all variables. Models were fitted using maximum likelihood during model comparison.

For each model we also calculated the repeatability of our random intercept terms (i.e. the amount of variance in hair cortisol concentration not explained by model fixed effects that can be attributed to consistent differences between provinces/years/months) with confidence intervals determined using parametric bootstrapping in the package rptR [53] with 5,000 iterations. We determined both the marginal R^2 (variance explained by fixed effects) and conditional R^2 (variance explained by fixed and random effects) following the procedure of Nakagawa and Schielzeth [54].

Results

HPLC analyses of hair glucocorticoids

Analysis of HPLC fractions from the hair extract pooled from 294 individuals confirms cortisol as the major glucocorticoid in hairs from the mongoose (Fig 1) eluting in fractions 13 and 14. That coincides with the position of authentic cortisol corresponding to 40% of total

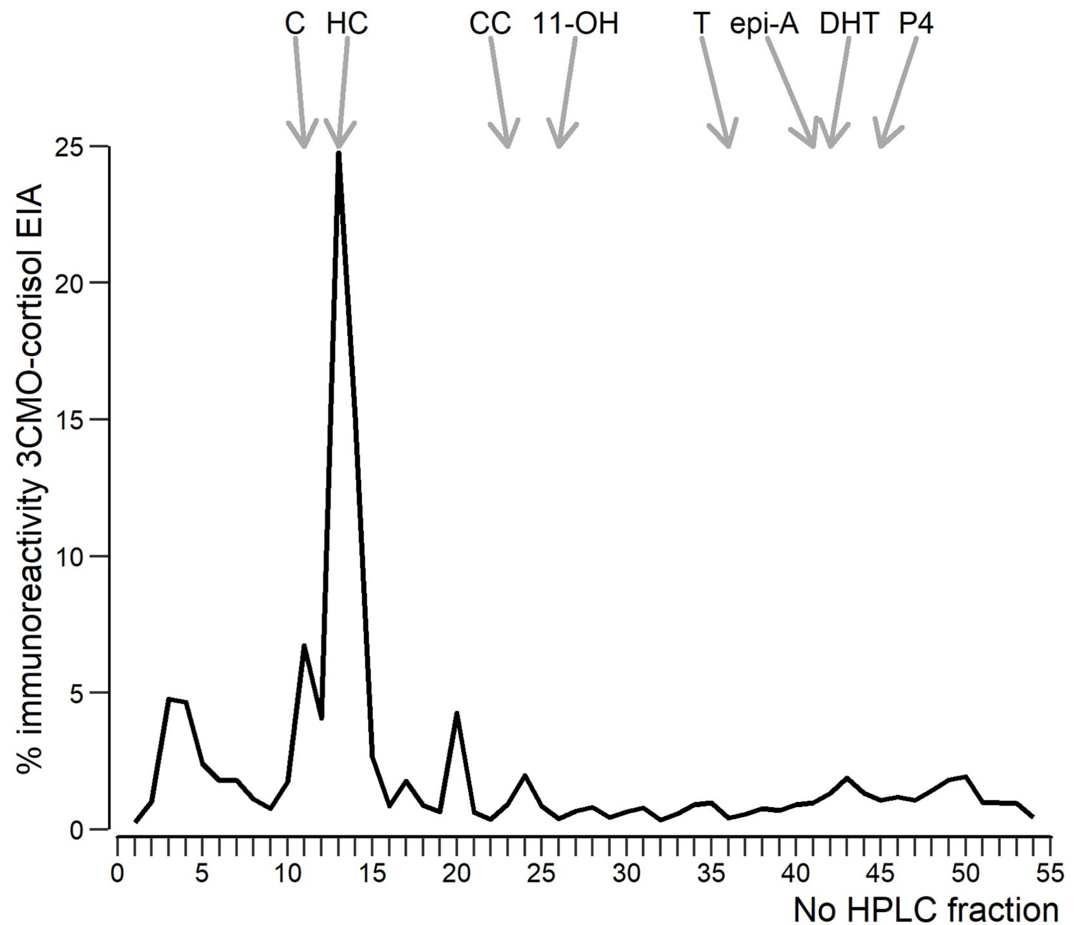

Fig 1. High performance liquid chromatography (reversed phase) separations of immunoreactive cortisol metabolites in pooled hair samples from Egyptian mongoose. The obtained fractions were analysed with a cortisol-3-CMO EIA. The elution positions of reference standards are indicated by arrows: 11: C (cortisone); 13/14: HC (cortisol); 23: CC (corticosterone); 26: 11-OH (11-hydroxyetiocholanolone); 36/37: T (testosterone); 41: epi-A (epi-androsterone); 42: DHT (dihydrotestosterone); 45: P4 (progesterone).

https://doi.org/10.1371/journal.pone.0221124.g001

immunoreactivity. Furthermore, three minor immunoreactive glucocorticoid metabolites peaks became visible in fractions 3–5, 11, and 20. From those, the immunoreactive compound in fraction 11 coincides with the position of authentic cortisone whereas the two remaining components co-elute with none of the steroid standards applied on column in previous runs. The most polar compound eluting in fractions 3–5 might indicate a cross-reacting glucocorticoid conjugate.

EIA of hair cortisol

The mean cortisol concentration detected in Egyptian mongoose guard hair was 19.99 ± 8.52 pg/mg (ranging from 8.07 pg/mg to 114.18 pg/mg). The inter-assay coefficients of variation were 10.78% for extracts containing low and 15.95% for extracts containing high concentrations of cortisol. The intra-assay coefficients were 6.72% (n = 16) for extracts containing low and 5.37% (n = 16) for extracts containing high concentrations of cortisol. The sensitivity of the assay was 0.40 pg/well. Hair cortisol concentrations of two animals which were obtained from different provinces and years were statistically compatible with outliers, but within

biologically plausible ranges according to ACTH stimulation tests done in other species. One was a sub-adult male collected in spring and the other was a non-breeding adult female collected in winter. There was no aspect of their data that allowed us to relate them and to explain the high level of cortisol.

Effects of age, sex, season, and storage time

There was no evidence for an interaction between sex and either season or age (Table 1), therefore all further results consider a model without interaction terms included. Hair cortisol concentration varied significantly with mongoose age, with early stage juveniles showing higher hair cortisol concentration than all other age cohorts (Fig 2a; Table 1). Hair cortisol concentration also differed significantly between sexes, with males showing higher hair cortisol concentration than females (Fig 2b; Table 1). There was also a significant change in hair cortisol with storage time, with lower hair cortisol concentration the longer the sample was stored (Fig 2c; Table 1). Hair cortisol concentration did not vary significantly between seasons, although mean hair cortisol concentration from summer samples tended to be lower than those in other seasons (Fig 3; Table 1). Parameter estimates were qualitatively similar between models fitted both with and without the two major outliers (Table 2).

The full model, with outliers removed, gave a marginal R^2 value of 0.19 (variance explained by fixed effects) and a conditional R^2 value of 0.39 (variance explained by fixed and random effects). Repeatability of all three random effects was low and could not be distinguished from zero in any case after parametric bootstrapping (Repeatability ± standard error: Province 0.09 ± 0.07; Year 0.09 ± 0.12; Month (within year) 0.06 ± 0.05).

Effect of female reproduction state

There was no evidence of an effect of reproductive state on hair cortisol in female mongoose (Table 1; Fig 4; Table 3). Effects of age, season and storage time were consistent with analysis conducted with both sexes (Table 3).

Discussion

This study validated an EIA for hair cortisol measurement and characterized its baseline variation in a wild population of Egyptian mongoose. Our analysis encompassed individuals of

Table 1. Significance of model terms calculated using parametric bootstrapped likelihood ratio tests.

Variable	Likelihood ratio test value	p-value
Interaction*	6.40	0.428
Storage	**9.51**	**0.020**
Sex	**5.05**	**0.028**
Season	5.71	0.187
Age	**19.38**	**0.001**
Reproductive state**	2.22	0.362

Likelihood ratio test value is the value of the likelihood ratio test value generated from the true data, which was then compared to likelihood ratio test values simulated with parametric bootstrapping.

*Note that the significance of interactions was determined by comparing a model with two interactions (sex and age, sex and season) to one with no interactions included.

**Significance of all terms except reproductive state are calculated using a model with data from both male and female mongoose. Significance of reproductive state was calculated using a model with data from females only (see section 2.5.2). Significant terms (α = 0.05) are in bold

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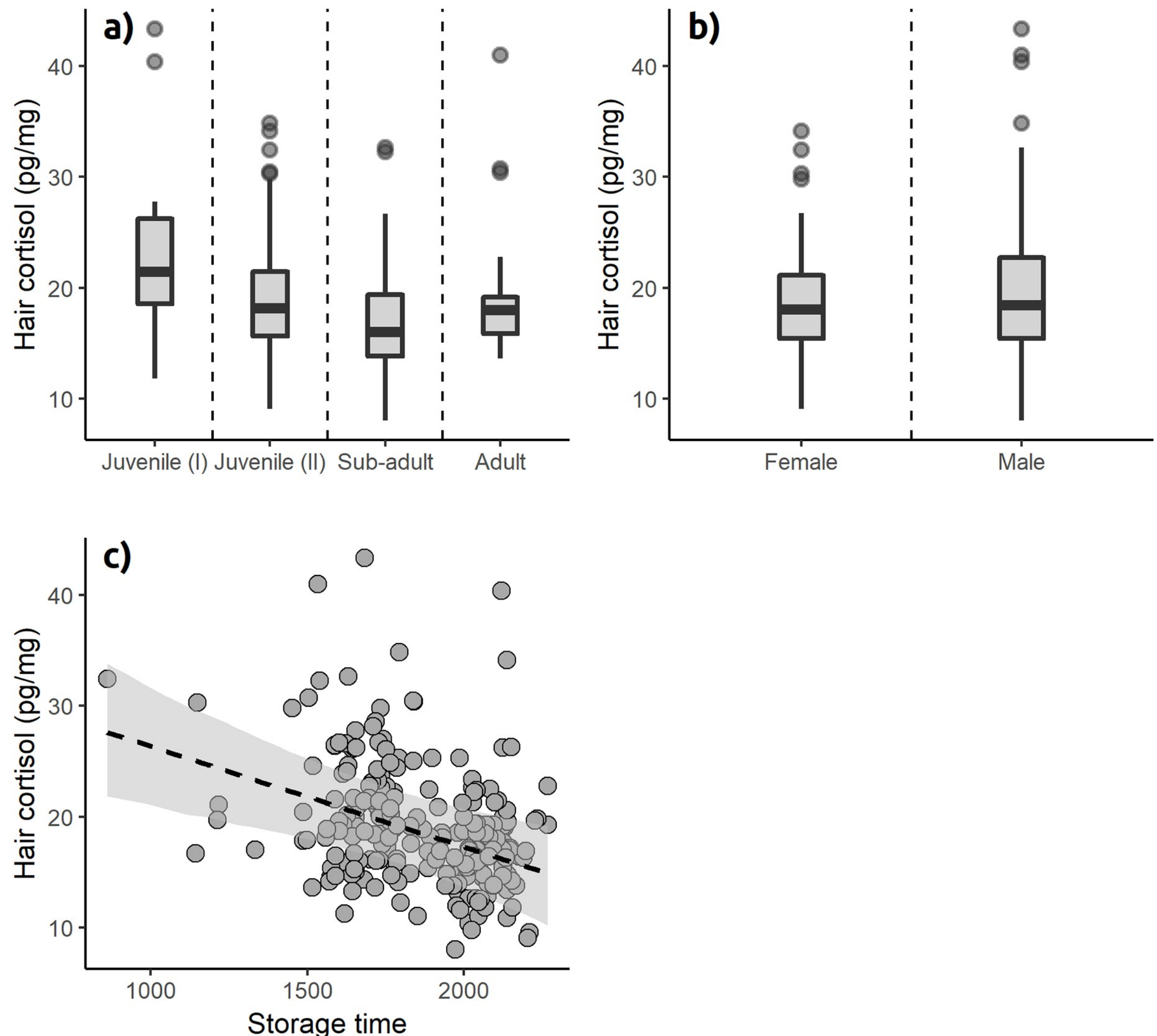

Fig 2. Variation in hair cortisol (pg/mg) in the Egyptian mongoose with a) age, b) sex, and c) storage time of hair samples (days). Hair cortisol concentration was higher in first stage juveniles than other age groups. Hair samples from males had higher cortisol concentration than females. Cortisol concentration was lower in hair samples stored for more days.

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both sexes and all ages, across a range of geographic, environmental and seasonal conditions that the species experiences in Portugal allowing us to account for spatial, temporal and biological factors that may contribute to hair cortisol variation. Our results showed that age, sex and storage time had an effect on hair cortisol, but season did not. By identifying which factors influence baseline hair cortisol in this wild population, we have established the basis for the application of hair cortisol measurement to understand the effect of natural and anthropogenic stressors.

Following methanol extraction, glucocorticoid metabolites (GCM) in a pooled hair sample were characterized by high-performance liquid chromatography (HPLC) and enzyme immunoassay (EIA). One major peak co-eluting with the cortisol standard was present. Besides cortisone, two unknown immunoreactivities were detected at positions not coinciding with one of our available steroid standards. This agrees with data in guinea pigs from Keckeis et al. [47],

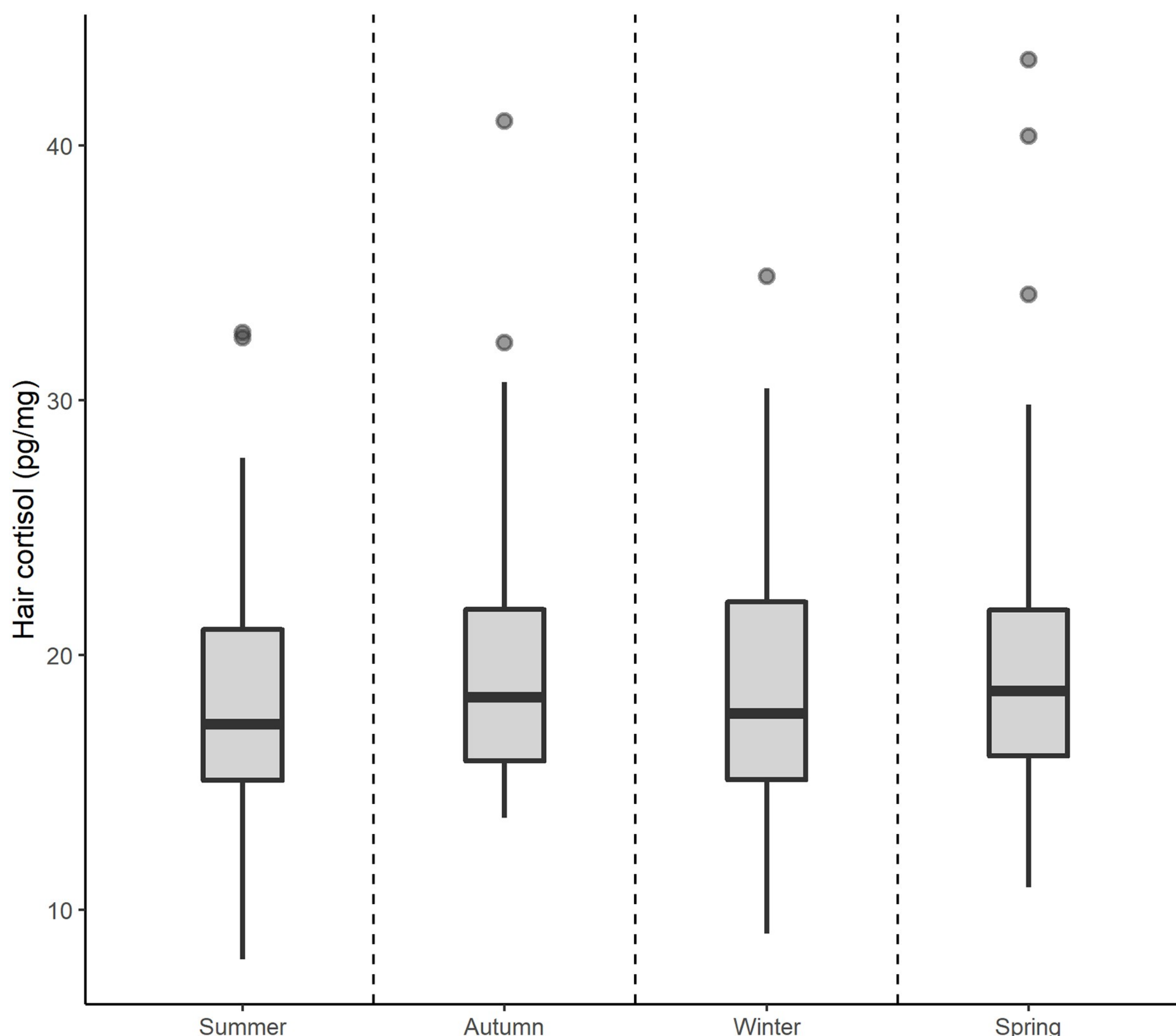

Fig 3. Variation in hair cortisol (pg/mg) in Egyptian mongoose collected in different seasons. Hair cortisol concentration was lower in samples taken in summer than other seasons, although this effect was not significant.

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Table 2. Effects of age, sex, season and storage time on hair cortisol concentration of Egyptian mongoose in two models fitted with and without two major outliers.

Variable	Parameter estimate (outliers removed)	[95% confidence interval]	Parameter estimate (outliers included)	[95% confidence interval]
Intercept	**33.96**	**[23.35/44.73]**	**32.05**	**[18.12/46.08]**
Juvenile (I)	**4.31**	**[2.22/6.41]**	**4.51**	**[0.73/8.22]**
Juvenile (II)	-0.50	[-2.46/1.46]	-0.60	[-4.11/2.85]
Sub-adult	-0.42	[-2.46/1.55]	1.29	[-2.29/4.9]
Autumn	1.63	[-0.83/4.1]	1.56	[-2.93/6.02]
Winter	2.29	[-0.63/5.19]	4.87	[-0.2/9.85]
Spring	**2.61**	**[0.26/4.99]**	3.01	[-1.41/7.41]
Male	**1.36**	**[0.1/2.61]**	1.35	[-0.86/3.55]
Storage time	**-0.01**	**[-0.02/-3e^{-3}]**	**-0.01**	**[-0.02/-7e^{-4}]**

The table shows parameter estimates of a general linear mixed effects model with 95% confidence intervals (estimated with parametric bootstrapping with 5,000 iterations). All parameter estimates where 95% confidence interval does not include 0 are in bold. Adult females in summer are used as the reference level.

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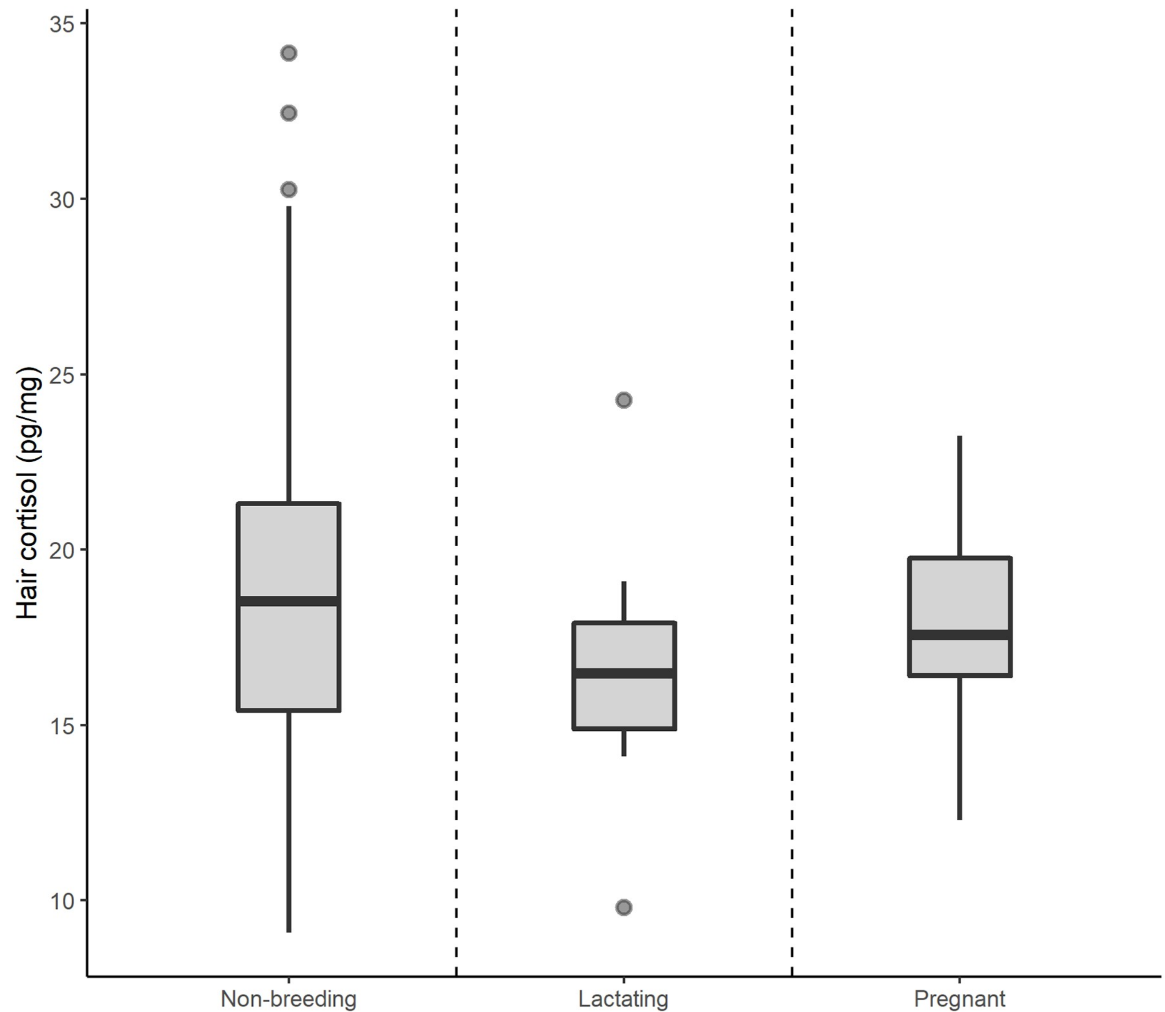

Fig 4. Variation in hair cortisol concentration in female Egyptian mongoose in different reproductive states. Hair cortisol concentrations were similar between reproductive states.

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who found cortisol, cortisone and corticosterone as well as unknown immunoreactivities, when applying EIAs for cortisol and cortisone. Particularly, our polar immunoreativities in fractions 3–5 were also found by these authors. Unknown cortisol-like immunoreactivities were also found in sheep by the same group [55], with different amounts depending on the selected EIA. Altogether our results revealed our cortisol assay is a suitable diagnostic tool for quantifying cortisol in hairs from the mongoose.

Age significantly influenced hair cortisol concentration in Egyptian mongoose, with early stage juveniles, between two-and-a-half and five-and-a-half months, exhibiting higher levels of hair cortisol than other age cohorts (Fig 2a). This age cohort includes the recently weaned mongoose pups [56] and is consistent with the post-weaning cortisol increase found in the southern elephant seal [33]. Elevated glucocorticoid secretion during infancy has been described using faecal glucocorticoid metabolite analysis in baboons [57] and hair cortisol in non-human primates [58] and humans [59]. However, our present findings are inconsistent with hair cortisol data from other wild carnivores, such as grizzly bears [12], polar bears [60] and the Canada lynx [14], where no effect of age was detected. This difference could be related

Table 3. Effects of age, season, storage time and reproductive state on hair cortisol concentration of female Egyptian mongoose.

Variable	Parameter estimate (outliers removed)	[95% confidence interval]
Intercept	**31.49**	**[24.14/38.77]**
Lactating	-1.81	[-5.11/1.51]
Pregnant	-1.32	[-3.79/1.27]
Juvenile (I)	**2.5763**	**[0.15/5.01]**
Juvenile (II)	-0.6448	[-3.16/1.83]
Sub-adult	-2.1557	[-4.52/0.19]
Autumn	-0.2291	[-2.56/2.05]
Winter	1.6513	[-0.81/4.06]
Spring	**2.2434**	**[0.14/4.30]**
Storage time	**-0.01**	**[-0.01/-4.6e^{-3}]**

Table shows parameter estimates of a general linear mixed effects model with 95% confidence intervals (estimated with parametric bootstrapping with 5,000 iterations). All parameter estimates where 95% confidence interval does not include 0 are in bold. Lactating adults in summer are used as the reference level.

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to differences in hair growth and moult patterns between species during their juvenile development. While diffusing into the hair during active growth, the amount of cortisol incorporated could be influenced by different types of juvenile coats and rapid growth and moult rates, and this effect could be even more pronounced in the presence of increased circulating cortisol levels. Unfortunately, we know very little about hair growth and moult patterns of wild species, including the Egyptian mongoose. An alternative explanation could be that age classification using dental growth allowed a finer scale of age classification in our study by separating age cohorts that are otherwise morphologically similar. On the other hand, bears and lynxes, as apex predators, are unlikely to be subject to predation or aggressive interspecific interactions. In comparison, the Egyptian mongoose, a meso-predator, experiences aggression and killing by sympatric carnivores like the Iberian lynx, wild domestic dogs [56,61,62] or large raptors [63], which could result in elevated cortisol levels. The characterization of hair growth and moult patterns and comparison of developmental patterns of hair cortisol in sympatric apex- and meso-predators would help clarify these questions. Analysis of hair cortisol in Iberian lynx, Egyptian mongoose, and other species of small carnivores in Portugal would provide an ideal system for such work.

Our data also support an effect of sex on hair cortisol concentration, with males showing higher cortisol concentration than females. This effect was not observed in previous studies in reindeer [9], grizzly bears [12] or Canada lynxes [14]. Investment in reproduction differs substantially between sexes in Egyptian mongoose and annual peaks in body condition and spleen weight are different between sexes and coincide with moments of investment in reproduction [45,46]. Egyptian mongoose males need to maintain and defend large territories [43] with frequent aggressive encounters with conspecifics [64] which might explain the observed sex differences in hair cortisol. On the other hand, the effect of sex could also be explained by the attenuation in HPA-axis reactivity in breeding females in order to avoid diverting resources from reproduction, as described in other species [65]. Either way, we would expect these effects to be seasonal, and therefore visible in the interaction of sex with season of our model; however, this was not the case. Indeed, there was no significant effect of season or the interaction of season with sex, which questions these explanations. It is possible that the effect is simply due to differences in hypothalamic-pituitary-adrenal axis function between sexes [32,66–68] in this species.

Season had no effect on hair cortisol levels in our study. However, a knowledge gap on hair cycles in wild mammals limits our ability to determine to which periods our hair cortisol values refer to. Wild terrestrial mammals in arctic and temperate climates usually undergo two yearly moults, one in spring and one in autumn, that do not overlap with periods of reproductive activity [69,70]. The retention of the previous pelage throughout reproduction could be the reason no changes in hair cortisol levels were found in our data during the reproductive period of the Egyptian mongoose. Hair cycles have been described previously in humans, domestic and laboratory species, pinnipeds and mustelids, but the variability in hair cycles even between closely related species [69] impedes extrapolation to the case of the Egyptian mongoose. Early observations do not support the existence of seasonal differences in coat length in this species in Israel [56]. Assuming this species moults at least once a year, we would expect to find an effect on hair cortisol due to changes in incorporation rate in the seasons when moults occurred, but we did not. This raises important questions regarding the interpretation of hair cortisol levels in wild mammals in general, and prompts research focusing on the effect of hair cycles in order to allow us to fully interpret our results on a temporal scale. While a confounder at this point, this information is a potential strength in the application of hair cortisol as a marker of stress once the effect of hair cycles is understood, because the signal of elevated cortisol levels during major life events might remain detectable for several months.

Storage time had a negative effect on the amount of cortisol retrieved from hair. Previous studies in animals have seen no influence of storage time on hair cortisol when intact hair is stored at room temperature for over one year [11,12], and based on a study in Peruvian mummies [71] it is presumed to remain detectable for longer periods. However, an effect of storage on grizzly bear hair stored in ground form was observed over a period as short as nine months [12], suggesting that hair integrity plays an important role. The longer duration of storage and the effect of freezing on hair integrity are possible explanations for the presence of this effect in our study. Our samples were stored between 863 to 2,266 days before processing, which four-fold exceeds the storage time assessed in grizzly bears [12] and dairy cattle [11]. Since the decrease in hair cortisol over time should occur very slowly, this small effect is more likely to be detected in studies focusing on longer periods and where a broad range of factors with potentially larger effects such as sex, age, geographic and temporal variation, are accounted for. Finally, part of the storage time of our samples was spent in the form of frozen cadavers before thawing and collection of hair samples, which could also have caused loss in hair integrity. The other possibility is the existence of a true variation in hair cortisol concentration of these animals due to changes in biotic or abiotc factors (e.g. predator-prey cycles) over the four years of the sampling period. This explanation was controlled for by the inclusion of month and year as random factors in model construction.

We expected lactating and pregnant females to exhibit higher baseline adrenocortical activity, and consequently more cortisol in hair. However, reproductive state was not significant in our female-only model. This could be explained by the small number of reproductively active females in our sample, to the difficulty in accurately detecting lactation or early pregnancies during dissection of previously frozen specimens, or simply by the fact that some females may have similarly high levels of hair cortisol due to factors we have not accounted for in our model. Despite non-significant, looking at the raw data (Fig 3), lactating and pregnant females tend to have lower hair cortisol concentration when compared to non-breeding females. According to studies in different mammal species, baseline HPA-axis activity is decreased during the initial period of pregnancy but steadily increases during the second and last thirds of pregnancy, peaking at parturition [65,72]. Meanwhile, HPA-axis reactivity to stress is supressed throughout the entire pregnancy [73]. A similar trend is seen during lactation, where an increase in baseline activity is accompanied by attenuated HPA-axis reactivity

[65,74]. Because hair incorporates blood cortisol from baseline levels and stress-induced responses altogether, our results suggest that, in breeding females, the net effect of the increase in baseline and supressed reactivity resulted in total cortisol levels similar to those of non-breeding females.

Conclusion

We investigated the variation of hair cortisol in a wild population of Egyptian mongoose in Portugal. Our results describe the baseline variation in hair cortisol in this population and highlight the importance of accounting for influences of age, sex and storage time when using hair cortisol. With this information, future studies should be able to apply hair cortisol measurements as a non-invasive technique to study effects of natural and anthropogenic stressors in wild mammals.

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Formal analysis: Alexandre Azevedo, Liam Bailey.

Funding acquisition: Katarina Jewgenow.

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Methodology: Alexandre Azevedo, Liam Bailey, Victor Bandeira, Martin Dehnhard, Katarina Jewgenow.

Project administration: Katarina Jewgenow.

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Supervision: Martin Dehnhard, Liliana de Sousa, Katarina Jewgenow.

Validation: Martin Dehnhard, Katarina Jewgenow.

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Writing – review & editing: Alexandre Azevedo, Liam Bailey, Victor Bandeira, Martin Dehnhard, Carlos Fonseca, Liliana de Sousa, Katarina Jewgenow.

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3. Article 2 – challenges in hair enzyme immunoassay validation

Jewgenow K, Azevedo A, Albrecht M, Kirschbaum C & Dehnhard M (2020). Hair cortisol analyses in different mammal species: choosing the wrong assay may lead to erroneous results. *Conservation Physiology*, 8(1), coaa009. https://doi.org/10.1093/conphys/coaa009

The aim of this research was to investigate if EIAs for hair cortisol measurement performed similarly in different species. For that purpose, we measured hair cortisol in Egyptian mongoose (*Herpestes ichneumon*), Iberian lynx (*Lynx pardinus*), Alpine marmot (*Marmota marmota*), Asiatic black bear (*Ursus thibetanus*), cheetah (*Acinonyx jubatus*) and spotted hyena (*Crocuta crocuta*) in identical extracts using two different EIAs (cortisol-3CMO EIA, cortisol-21-HS EIA). Next we determined glucocorticoid content in hair samples using “gold standard” LC-MS/MS, and analysed the EIA data in light of these results. Finally, we performed HPLC to determine the major immuno-reactive glucocorticoid components in the hair extracts in the six species and compare them with the elution positions of authentic steroids. Finally, we replicated the statistical analysis that had been previously performed on Egyptian mongoose data using the cortisol-3CMO EIA with the results of the cortisol-21-HS EIA, to investigate if the results were qualitatively similar.

Author contributions:

KJ: conception and design, analysis and interpretation, writing, review and editing; **AA**: design, laboratory analysis and data acquisition, writing (sections on Egyptian mongoose and Iberian lynx), analysis and interpretation, review and editing; MA: laboratory analysis and data acquisition, analysis; CK: laboratory analysis and data acquisition (LC-MS/MS), review and editing; MD: analysis and interpretation, writing, review and editing, supervision.

Results of the study:

1. In the six species studied, the cortisol-21-HS EIA resulted in hair cortisol measurements 2.3 to 12 times higher than the cortisol-3CMO EIA.

2. When compared with LC-MS/MS quantification, cortisol-3CMO EIA still resulted in an up to three-fold overestimation of hair cortisol.

3. Based on HPLC results, up to 70% of the immunoreactivity using the cortisol-21-HS EIA was due to unknown compounds that led to cortisol overestimation. In the case of the cortisol-3CMO EIA, major peaks of immunoreactivity were found in the cortisol position, followed by cortisone, which together were responsible for 32.1-67.4% of the reactivity.

4. In all species except the Asiatic black bear, both cortisol and cortisone were present in the hair samples. Cortisol-3CMO EIA measurement in these species was correlated with the sum of cortisol and cortisone values based on LC-MS/MS analysis.

5. In the Egyptian mongoose, the results of linear mixed effects models for data obtained with cortisol-3-CMO EIA had shown that age, sex and storage time had an effect on hair cortisol. In contrast, the same analysis performed on cortisol measurements with cortisol-HS-21 EIA revealed that only age had an effect, yielding strikingly different results.

6. Results of this study suggest that any EIA to measure hair glucocorticoids should undergo testing for its specificity towards the extracted hair components, as choosing the wrong assay can lead to erroneous results.

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Research article

Hair cortisol analyses in different mammal species: choosing the wrong assay may lead to erroneous results

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Wild animals are faced with a broad range of environmental stressors and research is needed to better understand their effect on populations. Hormone analysis based on enzyme immunoassays (EIAs) can provide valuable information on adrenocortical activity (stress), and assessment of cortisol in hair may allow the quantification of cortisol production. To validate hair hormone analysis, we compared two EIAs based on antibodies against cortisol-3-CMO-BSA and cortisol-21-HS-BSA for hair glucocorticoid (hGC) measurements in Egyptian mongoose, Iberian lynx, Alpine marmot, Asiatic black bear, spotted hyena and cheetah, with results obtained by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) measurements. Both EIAs were also characterized by HPLC immunograms. Our results revealed that the cortisol-21-HS EIA measured 2.3- to 12-fold higher hGC concentrations than the cortisol-3-CMO assay. In dependence of the species, high-performance liquid chromatography (HPLC) immunograms showed that up to 70% of immunoreactivities determined by the cortisol-21-HS constituted of unknown unpolar compounds leading to an overestimation of hGC. The cortisol-3-CMO EIA expressed a better specificity, with 32.1–67.4% of immunoreactivity represented by cortisol and cortisone. The LC-MS/MS analyses (gold standard) revealed that the cortisol-3-CMO EIA also resulted in an (up to 3-fold) overestimation of hGC, but EIA results were correlated with LC-MS/MS in the mongoose, the lynx, the spotted hyena and the marmot. No correlation was obtained for Asiatic black bears. As a result of our study, we strongly recommend to test any cortisol EIA for its specificity towards extracted hair components. In all analyzed species, except the Asiatic black bear, cortisone and cortisol were simultaneously present in hair extracts; consequently, an appropriate EIA should cross-react to these two glucocorticoid hormones and express negligible affinity towards substances with less polarity than corticosterone. Choosing the wrong EIA for hGC analyses may lead to overestimations of hGC or—in the worst case—to results that do not mirror real adrenocortical activity.

Key words: glucocorticoids, hair, HPLC, immunogram, LC-MS/MS

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Introduction

Wild animals are faced with a broad range of environmental stressors, and one physiological process helping vertebrates to cope with any challenge is the stimulation of the hypothalamic pituitary axis (HPA) leading to the release of glucocorticoid (GC; corticosterone/cortisol) hormones. These metabolic hormones not only are involved in the maintenance of energetic balance but also act as key components of the acute stress response. Baseline levels of GC in free-living animals may vary during development and throughout the year. Thus, annually varying GC levels reflect the physiological response of animals to meet seasonal needs, which are thought to be different between sexes (Romero, 2002; Bauer *et al.*, 2014).

In case of acute stress events, however, increased levels of GC are essential to shift energy towards the functions demanded to rapidly cope with the stressor (amongst others dominance behaviour, intra- and inter-species competition, escape from predators and human–wildlife conflicts) (McEwen *et al.*, 2003). Usually, these changes are of limited duration, and thereafter GC levels return to baseline, but if adverse environmental conditions act permanently, continuously elevated GC secretion may cause deleterious consequences by altering individual fitness, immune function and reproductive success (Terio *et al.*, 1999; Tilbrook *et al.*, 1999; Ludwig *et al.*, 2019) and eventually reduce survival (Wingfield *et al.*, 2003). Thus, the possible impact of an environmental stressor, either of anthropogenic or of natural origin on an animal's fitness, can only be detected if physiological baselines are known and reliable measurements of GC are performed on a regular and large-scale basis.

Determination of cortisol as an indicator of the hypothalamic–pituitary–adrenal (adrenocortical) activity is often used to measure stress. Since serum GC concentrations are characterized by diurnal pulsatile fluctuations and capture/restraint for blood collection induce increases of GC concentration within a few minutes (Carnes *et al.*, 1988; Sheriff *et al.*, 2011), alternative approaches that use matrices like urine, faeces or hairs have been developed and validated for wild animals.

Urine and faeces can be collected non-invasively from terrestrial mammals without causing stress from capture, restraint and venipuncture procedures, mirroring adrenocortical (stress) status of the preceding hours or days. Urine, however, requires special sampling procedures in captive animals and is almost impossible in free-ranging animals. Faecal samples are easy to obtain from wild animals and DNA-based technologies can be applied (Sheriff *et al.*, 2011) to assign them to a species or even to individuals (Mohd Salleh *et al.*, 2017; Mengulluoglu *et al.*, 2019). In particular, faecal glucocorticoid metabolites (fGMs) are described as reliable indicators of GC excreted by the adrenal glands during the previous 12–24 h (depending on defecation rates). For some species, the delay may be even longer (e.g. reptiles). They are less affected by episodic fluctuations or the pulsatility of hormone secretion and are therefore useful to evaluate adrenal activity in an integrated manner (Sheriff *et al.*, 2011; Palme, 2019). However, successful measurement of fGM relies on the development of immunoassays that detect the species-specific hormone metabolites of cortisol or corticosterone. The native hormones are, if at all, represented in minor quantities in faecal samples, as has been shown, e.g. for carnivores (Young *et al.*, 2004). In most cases, fGM still cross-react with antibodies primarily produced to measure the respective unmetabolized GC in the plasma. However, experiments dealing with the physiological and biological validity of fGM analyses are essential to prove whether an immunoassay is able to reflect an animal's endocrine status. The most widely used approach is to stimulate adrenocortical activity with an adrenocorticotropic hormone (ACTH) challenge test or to measure an increase of fGM in response to a known stressor experienced by the animal (Touma *et al.*, 2004; Young *et al.*, 2004).

In contrast to fGM, assessment of cortisol in hair may reflect circulating cortisol levels over extended time periods, although the mechanism of incorporation is still a matter of debate. It is suggested that cortisol is incorporated into hairs by passive diffusion from the bloodstream and therefore may serve as a unique biomarker of HPA activity providing an integrated value of circulating hormones covering several weeks or even months (Davenport *et al.*, 2006; Accorsi *et al.*, 2008; Macbeth *et al.*, 2010; Ashley *et al.*, 2011; Gonzalez-de-la-Vara *et al.*, 2011; Terwissen *et al.*, 2013; Mastromonaco *et al.*, 2014). Consequently, hair cortisol is thought to be insensitive to the impact of sampling procedures, ultradian rhythms and the effect of any acute stressor, which certainly offers great potential as an indicator of chronic stress. However, a recent radio-metabolism study using labelled cortisol demands a cautious interpretation of the retrospective time frame to record stress in relation to the hair growth rate (Kapoor *et al.*, 2018). The authors also showed that radiolabeled cortisol was incorporated into hair of rhesus monkey as cortisol and cortisone and as other unknown metabolites, whereas in the guinea pig radiolabeled cortisol was incorporated mainly as cortisone (Keckeis *et al.*, 2012). Raul et *al.* (2004) suggested for human that the enzyme 11β-hydroxysteroid-dehydrogenase (HSD) (in)activates GCs converting cortisol to cortisone, and a conversion of cortisol to cortisone before its incorporation in hair (Raul *et al.*, 2004). Thus, in addition to cortisol, cortisone may also be regarded as a useful biomarker for stress research.

The authentic hormones should be detectable when applying an immunoassay. However, the application of antibody-based methods requires a thoughtful validation before they can provide useful information on the effect of stressors on animals. For example, the body regions from which samples are collected may influence the concentration of cortisol present in hair (Terwissen *et al.*, 2013; Acker *et al.*, 2018). Mechanical irritation by scratching might enhance local hair cortisol production as well (Salaberger *et al.*, 2016). Moreover, hair growth pattern might be highly

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variable between individuals, which may affect between subject comparisons (Kapoor *et al.,* 2018). In addition, the precise mechanisms by which lipophilic steroid hormones are incorporated into hair are still not fully understood. *In vitro*, an equivalent of the central HPA in the hair follicle of humans has been demonstrated (Ito *et al*., 2005). Data from a radiometabolism study performed in guinea pigs (Keckeis *et al*., 2012) demonstrated only little amounts of injected 3H-cortisol in the hair matrix, but surprisingly high amounts of unlabeled cortisol, assuming a local synthesis and quite likely an autonomous production of cortisol (Cirillo *et al*., 2011). In addition, reversed-phase high-performance liquid chromatography (HPLC) of hair samples from guinea pig and wool samples from sheep demonstrated-depending on the applied GC enzyme immunoassay (EIA)-the presence of cortisol, cortisone and corticosterone as well as of several unknown cortisol immunoreactivities (Keckeis *et al*., 2012, Salaberger *et al.,* 2016).

These unknown steroid hormones may hamper the determination of GC in hair samples (hGC) leading to an overestimation of GC content in hair samples due to unforeseeable cross-reactivities with the antibody. The application of high-performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) for exact quantification of hGC is therefore preferable to any immune assay. It combines the physical separation of liquid chromatography (LC) paired with the specificity of mass analysis capabilities of tandem mass spectrometry (MS/MS) allowing the sensitive detection and quantification of multiple compounds. Until now, only a few LC–MS/MS methods for the simultaneous identification and quantitation of steroids in hair have been described (Stalder *et al.,* 2013). In contrast to an EIA, LC-MS/MS equipment is quite expensive and demands high analytical skills. Therefore, it is often unavailable to smaller laboratories and, in particular, to researchers working on wildlife. For them, immunoassays are still the method of choice.

We recently validated an EIA to determine hair cortisol concentrations in wild caught Egyptian mongoose (Azevedo *et al*., 2019). In the present follow-up study, we applied two different cortisol EIA, and obtained different cortisol concentrations in identical hair extracts in the mongoose and five other wildlife species. By conducting HPLC immunograms, we determined the immunoreactive components in hairs and related them to known steroids, in particular to GC. This allowed us to identify the predominant hGC component in the six species. Finally, we assessed the steroid composition by LC-MS/MS analyses (gold standard) and analyzed the EIA quantification in light of the LC-MS/MS results.

Materials and methods

All chemical reagents were purchased from Sigma-Aldrich (Taufkirchen, Germany) unless stated otherwise and were of the highest purity available.

Sample collection

Hair samples were obtained from six mammalian species in the framework of different research programs: Egyptian mongoose (*Herpestes ichneumon*), Iberian lynx (*Lynx pardinus*), cheetah (*Acinonyx jubatus*), Alpine marmot (*Marmota marmota*), Asiatic black bear (*Ursus thibetanus*) and the spotted hyena (*Crocuta crocuta*).

Hair samples from the individual Egyptian mongoose (*Herpestes ichneumon*; $n = 294$; 140 males and 154 females; 147 adults and 97 juveniles and sub-adults) were collected from hunting activities (Bandeira *et al*., 2016) and were previously used to determine baseline variation in hair cortisol levels (Azevedo *et al*., 2019). Following death, specimens were frozen at −20°C until the date of sample collection. After thawing, hair was clipped with scissors from a standard area between the shoulders and stored in paper envelopes until further processing (Azevedo *et al.,* 2019). Samples from individual Iberian lynx (*Lynx pardinus*; 93 adult females, 6 adult males) were obtained during routine health checks within the Iberian lynx conservation breeding program in Spain and Portugal. The hair was clipped from the inner surface of hind legs.

All other hair samples were obtained from the IZW hair collection compiled over several years. Hair samples from 20 alpine marmot (*M. marmota*) were clipped from an area between shoulders (5 adult males, 4 adult female, 11 juveniles) housed at the Feldstation Niederfinow 70 km north of Berlin. The samples from adult spotted hyenas (*C. crocuta;* $n = 7$; 2 males, 5 females) and from the one adult male cheetah were collected from the tail region, whereas those from adult Asiatic black bears (*U. thibetanus*; $n = 14$; 9 males, 5 females) were obtained from the inner surface of hind legs. All hair samples were stored in envelopes at room temperature.

From all Iberian lynx and Egyptian mongoose hair samples, an aliquot of 20 mg was separated and washed twice with 2 ml of 90% methanol by vortexing for 5–10 s to remove surface contaminations. Thereafter, the samples were dried for 1 h at 70°C and aliquots (∼10 mg) from each Egyptian mongoose ($n = 294$) and female Iberian lynx ($n = 93$) were taken and milled to a fine power with ceramic beads in a tissue homogenizer as described before (Azevedo *et al*., 2019). Then, 400 μl of 90% methanol was added to the powder and shaken at room temperature for 30 min. Following centrifugation (3 min, 1000×g), the supernatant was collected and transferred into a new tube, diluted 1:2 with water and kept frozen until EIA analysis and the preparation of HPLC immunograms. From hair samples of 14 mongooses (7 males, 7 females) and 12 lynxes (6 males, 6 females), additional extracts were produced for LC-MS/MS analyses (see below) following the same protocol.

For hair samples from the Asiatic black bear, the alpine marmot, the cheetah and the spotted hyena, the washing procedure was modified utilizing aliquots of 50–60 mg that

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were washed twice with 4.5 ml of 90% methanol. Thereafter, the samples were extracted as described by Carlitz *et al.* 2014. In brief, before washing and drying, hairs were cut into 10-mm pieces and afterwards 50 mg of the dried hairs were incubated with 1.8 ml of methanol for 18 h at 45°C. Subsequently, the extract was aspirated, dried for 20 min at 60°C under a constant stream of nitrogen and resolved in 1 ml 90% methanol. An aliquot of 100 μl was diluted 1:2 with water for EIA and kept frozen. The remaining 900 μl was subjected to LC-MS/MS analyses or were pooled for HPLC-immunograms (see below).

GC analyses

We used two in-house immunoassays based on polyclonal antibodies (rabbit) directed against (i) cortisol-3-CMO-BSA and (ii) cortisol-21-hemisuccinate (HS)-BSA. The corresponding label of cortisol were cortisol-3-CMO-peroxidase and cortisol-21-HS-peroxidase as previously described in (Ludwig *et al.*, 2013) and in (Kelm *et al.*, 2016), respectively. The antibody cross-reactivities of the cortisol-3-CMO assay were as follows: cortisol, 100%; cortisone, 19.5%; corticosterone, 6.3%; desoxycorticosterone, 0.1%; progesterone, 0.1%; estradiol, 0.1%; testosterone, 0.1%. Those of the cortisol-21-HS EIA were cortisol 100%; cortisone, 17.5%; progesterone, 13.2%; corticosterone, 10.4%; desoxycorticosterone, 7.9%; estradiol, 0.1%. The principle of our in-house EIA procedure has been described in detail in Finkenwirth *et al.* (2010). All EIA measurements were conducted in 20 μl duplicates and results expressed as pg/mg hair weight.

For both EIA applied, serial dilutions of hair extracts showed parallelism to the cortisol standard with no significant difference in slopes ($P > 0.05$). The sensitivity of the assays was 0.40 pg/well. The inter-assay and intra-assay coefficients of variation were determined on two pooled extracts. For cortisol-3-CMO EIA, the pools contained 0.25 and 0.6 ng/ml cortisol. Inter-assay and intra-assay coefficients of variation were 11.3% ($n = 7$) and 9.5% ($n = 7$), and 6.7% ($n = 16$) and 5.4% ($n = 16$), respectively. For the cortisol-21-HS assay, the inter-assay and intra-assay coefficients of variation were determined on two different extraction pools, which were composed of 0.25 ng/ml and 0.5 ng/ml cortisol. The corresponding inter- and intra-assay variations were 14.6% ($n = 7$) and 9.8% ($n = 8$), and 12.8% ($n = 16$) and 5.9% ($n = 16$), respectively.

High-performance liquid chromatography

To characterize the compounds detected by the respective EIA, we ran HPLC immunograms from pooled hair extracts that were obtained using two different extraction procedures (see above). From the hair extracts of 294 mongooses and 93 female lynxes, aliquots of 150 and 460 μl of each extract were pooled corresponding to ~550 and 530 mg of hairs, respectively. For the marmots ($n = 20$) and black bears ($n = 14$), 50 mg hair of each individual was extracted as described and 900-μl aliquots of each extract were combined, resulting in a pool of ~900 and 630 mg of hairs, respectively. From each individual hyena samples ($n = 7$), two different hair aliquots of 50 mg were extracted, whereas from the one cheetah 20 hair aliquots of 50 mg were taken. From each extraction, 900 μl was pooled, so the pools for hyena and cheetah represented ~630 and 700 mg of hairs, respectively. All pools were purified on Octadecyl C18 columns (0.5 ml, J.T. Baker, BAKERBOND SPE^{TM} 7020-01) as described in Dehnhard *et al.* (2010) and Azevedo *et al.* (2019). Eluates were evaporated in a heater at 55°C under nitrogen and reconstituted in 200 μl of 40% methanol. One hundred to 150 μl portions of purified hair extract were used for HPLC analysis on a reverse-phase Ultrasep ES100/RP-18/6 μm HPLC column (4 × 250 mm, Sepserv, Berlin). Compounds were separated using a methanol + water mixture with the following gradient: 60% methanol over 5 min, 60–90% methanol over 10 min and 90–100% methanol over another 10 min. The flow rate was 1 ml/min. Fractions of 0.33 ml were collected at 20-s intervals over a period of 25 min. All fractions were lyophilized and re-suspended in 200 μl 40% methanol before 20-μl aliquots were determined by both cortisol-EIA. The elution positions of native cortisone, cortisol, corticosterone, 11-hydroxyetiocholanolone, testosterone (T), epi-androsterone, dihydrotestosterone (DHT) and progesterone (P4) on this column had been previously determined in separate HPLC runs (Ludwig *et al.*, 2013; Pribbenow *et al.*, 2015).

Liquid chromatography coupled with tandem mass spectrometry

Hair extracts from individual mongooses ($n = 14$), Iberian lynxes ($n = 12$), marmots ($n = 20$), Asiatic black bears ($n = 14$) and spotted hyenas ($n = 7$) were adjusted to an absolute content about 150 pg cortisol. To achieve this, cortisol concentrations of extracts were determined by 3-CMO EIA, and the required volume of each species was determined according to the mean cortisol concentration (Table 2). Thus, for mongoose 300 μl (~7.5 mg hair), for Iberian lynx 200 μl (~5 mg hair), for marmots 600 μl (~30 mg hair) and for black bears 800 μl (~40 mg hair) of each extract were transferred to 1.5-ml reaction tubes, dried down for 90 min at 60°C under a constant stream of nitrogen and dissolved in 225 μl 50% methanol (LC-MS/MS grade). The samples were sent to the Department of Biopsychology, Technical University of Dresden, Germany, for LC-MS/MS analyses. Details on liquid chromatography methodology and mass spectrometric conditions are described in Carlitz *et al.* (2016) and Gao *et al.* (2013).

Statistical methods

Determination of mean values ± SD, comparison of cortisol amounts between both EIA as well regression analysis were

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Table 1: hGC concentrations in five different mammalian species determined by two different cortisol EIA, a cortisol-3-CMO and a cortisol-21-HS. Represented are means ± SD. For correlation analysis, Pearson's correlation coefficients (*r*) were determined

Species (Number)	Cortisol-21-HS in pg/mg Mean ± SD (Range)	Cortisol-3-CMO in pg/mg Mean ± SD (Range)	Relation	correlation coefficient *P* value
Egyptian mongoose (294)	159.7 ± 46.4 (61.9–394.6)	20.0 + 8.5 (0.0–114.2)	8: 1	$r = 0.324$ **$P = 0.000$**
Iberian lynx (12)	87.7 ± 46.9 (32.2–174.3)	37.3 ± 13.2 (21.1–60.5)	2.3: 1	$r = 0.863$ **$P = 0.000$**
Alpine marmot (20)	60.6 + 18.6 (36.3–111.5)	6.0 + 1.9 (4.1–11.5)	10: 1	$r = 0.190$ $P = 0.423$
Asiatic black bear (14)	44.6 + 20.2 (15.5–76.2)	3.7 ± 2.3 (0.8–10.6)	12: 1	$r = 0.619$ **$P = 0.009$**
Spotted hyena (7)	28.9 + 16.8 (18.0–60.7)	12.1 ± 2.7 (6.6–17.5)	2.3: 1	$r = 0.507$ $P = 0.246$

Differences in mean for cortisol levels determined by cortisol-21-HS and cortisol-3-CMO EIA were highly significant ($P < 0.001$) for the Egyptian mongoose, the Iberian lynx, the marmot and the Asiatic black bear, whereas in case of the spotted hyena the difference was significant ($P = 0.036$). The respective *t* values (degree of freedom) in paired *T* test were 56.039 (293), −4.833 (11), −13.400 (17), 7.830 (13) and −2.684 (6) for the mongoose, lynx, marmot, black bear and hyena, respectively.

performed using IBM SPSS Statistics 24 (SPSS Inc., IBM, Armonk, USA). Significance was set to $P < 0.05$. Tukey box plots were produced by SigmaPlot for Windows (Version 10.0).

Paired *t* test for comparison of means based on two-tailed *P* values was applied after testing for normality. Pearson correlation coefficients were calculated to detect the relation between the two cortisol EIA (Supplement Table 1). Regression analysis was also performed between the hGC amount determined by LC-MS/MS (sum of cortisol and cortisone) in relation to the amount determined by EIA (Supplement Table 2).

As described before in Azevedo *et al.* (2019) for the results of cortisol-3-CMO EIA, the statistical analysis of the effects of age, season, sex and storage on hair cortisol measured with cortisol-21-HS EIA was performed in R (v3.5.1) using linear mixed effects models with a Gaussian error distribution using the package lme4 (Bates *et al.* 2015), and variable significance ($\alpha = 0.05$) was determined with parametric bootstrapped likelihood ratio tests with 5000 iterations using the package pbkrtest (Halekoh and Højsgaard, 2014).

Linear mixed effects models and likelihood ratio tests were performed on data without outliers for cortisol-3-CMO EIA (taken from Azevedo *et al.* 2019) and 21-HS-cortisol EIA. Values four times larger than median hair cortisol and almost twice as high as the next highest measurement were defined as outlier. Four-fold increases in hair cortisol have previously been observed in response to repeated ACTH challenge in dairy cattle (Gonzalez-de-la-Vara *et al.* 2011) and eastern chipmunks (Mastromonaco *et al.* 2014), and so these values may be biologically plausible in situations of chronic and severe stress.

Results

Determination of cortisol content in hair samples using EIA

Within a framework of other projects, 294 hair samples from Egyptian mongoose were analyzed with both cortisol EIA revealing significant differences between both EIA (Table 1, Supplement Table 1, paired *t* test: $t = 56.039$, $df = 293$, $P < 0.0001$). The mean cortisol content determined with cortisol-21-HS EIA was about eight times higher compared to the results obtained with the cortisol-3-CMO assay; both data sets were correlated with each other (Table 1, Fig. 1, $r = 0.324$, $P < 0.0001$). By applying linear mixed fixed models for data obtained with cortisol-3-CMO EIA it was shown that age, sex and storage time had an effect on hair cortisol, but season did not. The full data analysis was reported in Azevedo *et al.* (2019). In contrast, the same analysis performed on hGC concentrations determined with cortisol-HS-21 EIA revealed that only age had an effect (Table 2, Supplement Table 3, Supplement Fig. 1).

In the Iberian lynx and the spotted hyena (Table 1, Supplement Table 2), the difference between the two cortisol EIA was less pronounced with less than half cortisol determined when using the cortisol-3-CMO assay (lynx: $t = -4.833$, $df = 11$, $P < 0.0001$; hyena: $t = -2.684$, $df = 6$, $P = 0.036$). From the samples from marmots and Asiatic black bears (Table 1, Supplement Table 2), we also obtained significantly different hGC measurements between the two EIA. Analyses with the cortisol-21-HS EIA revealed 10- and 12-fold higher hGC concentrations in the marmot ($t = -13.400$, $df = 19$, $P < 0.0001$) and the black bear ($t = 7.830$, $df = 13$ $P < 0.0001$), respectively. Although being significantly

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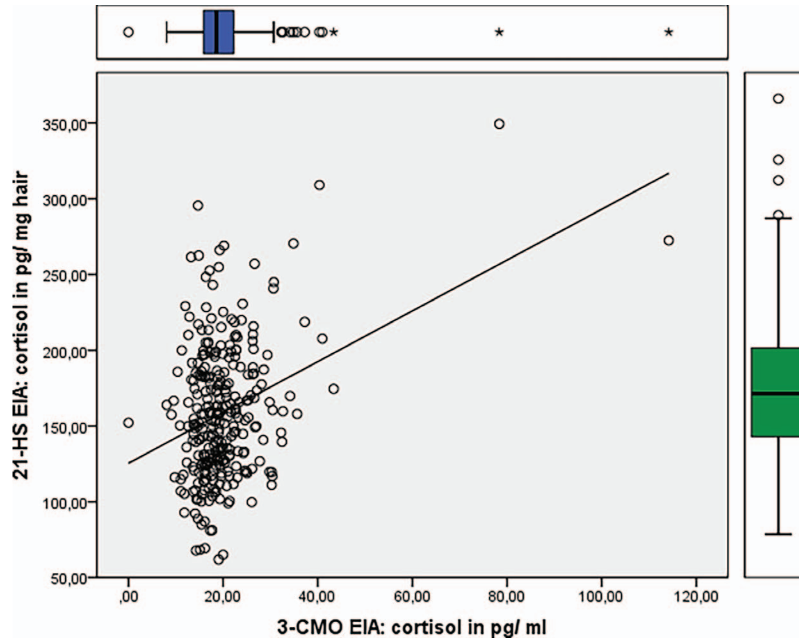

Figure 1: X-Y-diagram of cortisol data determined with 3-CMO EIA versus 11-HS EIA for all samples from mongoose, combined with box-plots of both EIA.

Table 2: Significance of model terms age, season, sex and storage time, calculated according to Azevedo *et al*. (2019) using parametric bootstrapped likelihood ratio tests, with results of cortisol-21-HS and cortisol-3-CMO EIAs, respectively

	Cortisol-21-HS		Cortisol-3-CMO	
Variable	Likelihood ratio test value	*P* value	Likelihood ratio test value	*P* value
Age	**3.052**	**0.029**	19.38	**0.001**
Season	2.039	0.109	5.71	0.187
Sex	0.086	0.769	**5.05**	**0.028**
Storage time	0.180	0.671	**9.51**	**0.020**

Likelihood ratio test value is the value of the likelihood ratio test value generated from the true data, which was then compared to likelihood ratio test values simulated with parametric bootstrapping. Significant terms (α = 0.05) are in bold.

different between the EIAs, results of both EIA were still correlated in the Egyptian mongoose, the Iberian lynx and the Asiatic black bear, whereas no relation between both EIA was found in alpine marmots and spotted hyenas (Table 1).

LC-MS/MS analyses

LC-MS/MS allows the selective detection of different GCs in hairs combining the physical separation of liquid chromatography (HPLC) paired with the specificity of tandem mass spectrometry (MS/MS) and was therefore used to assess the results obtained by the two different EIA. In our study, quantitative LC-MS/MS determinations of cortisone, cortisol and corticosterone were carried out in a total of 68 hair samples from the above mentioned species (limit of detection [LOD] = 0.32 pg/mg, limit of quantification [LOQ] = 1.05 pg/mg). In all samples, the cortisol content was determined by both EIA as well (Table 3, Supplement Table 2) making species differences apparent.

Highest cortisone and cortisol concentrations were found in the lynx followed by lower concentrations in the mongoose and the hyena. Mean cortisol concentrations below 1.0 pg/mg were obtained in the marmot and the black bear. Moreover, cortisol levels in 9, 3 and 10 samples were below detection limit in the mongoose, the marmot and the black bear, respectively. Except from the black bear, low corticosterone concentrations were obtained in all species and were completely undetectable in the lynx and the marmot. Although almost close to detection level, corticosterone was measurable in all black bear samples. Whether corticosterone represents the main hGC in the black bear needs to be confirmed.

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Table 3: hGC concentrations achieved by comparative LC-MS/MS determination of cortisone, cortisol and corticosterone in a total of 68 hair samples from six mammal species. In addition, cortisol concentrations were determined by both EIA, cortisol-3-CMO and cortisol-21-HS, respectively. Represented are means ± SD. For correlation analysis, Pearson's correlation coefficients (*r*) were determined between the sum of cortisol+cortisone and results of both EIA, respectively

Species (Number)	LC-MS/MS				hGC by EIA		Correlation coefficient; *P*-value			
	Cortisone (pg/mg)	Cortisol (pg/mg)	Corticosterone (pg/mg)	Cortisone+ cortisol (pg/mg)	21-HS EIA (pg/mg)	3-CMO EIA (pg/mg)	21-HS EIA vs. cortisol+cortisone		3-CMO EIA vs. cortisol+cortisone	
Egyptian mongoose (14)	6.11 ± 3.66	5.31 ± 9.39	1.81 ± 1.95	11.43 ± 12.63	193 ± 68.6	20.6 ± 15.0	r = 0.691	***P* = 0.000**	r = 0.914	***P* = 0.000**
Iberian lynx (12)	24.82 ± 9.58	13.35 ± 7.80	0.00	38.16 ± 12.27	87.8 ± 46.9	37.3 ± 13.2	r = 0.799	***P* = 0.001**	r = 0.855	***P* = 0.000**
Spotted hyena (7)	3.03 ± 1.21	4.41 ± 1.33	0.36 ± 0.62	7.44 ± 2.24	28.9 ± 16.8	13.6 ± 4.1	r = 0.5	*P* = 0.127	r = 0.807	***P* = 0.014**
Alpine marmot (20)	0.90 ± 0.65	1.14 ± 1.47	0.00	2.03 ± 2.1	60.6 ± 18.6	6.04 ± 1.92	r = −0.087	*P* = 0.358	r = 0.847	***P* = 0.000**
Asiatic black bear (14)	0.36 ± 0.13	0.06 ± 0.10	2.02 ± 1.18	0.42 ± 0.13	50.8 ± 22.6	3.7 ± 2.4	r = −0.252	*P* = 0.193	r = 0.10	*P* = 0.367
Cheetah (1)	7.2	3.7	1.2	10.9	55.5	13.8	-	-	-	-

Comparison between cortisol EIA and LC-MS/MS analyses

Altogether, LC-MS/MS analyses revealed similar species differences compared to the analyses with both EIA (Table 3, Fig. 2). In the lynx, the highest amounts of hGC were detected, and the range of LC-MS/MS concentrations expressed as the sum of cortisone + cortisol (38.16 ± 12.27 pg/mg) matched with the mean values obtained by the 3-CMO EIA (37.3 ± 13.2 pg/mg, Table 3).

In hair samples of mongoose (Table 3), the LC-MS/MS cortisone + cortisol content was determined to be 11.43 ± 12.63 pg/mg. In comparison, the 3-CMO EIA overestimated hGC twice (20.6 ± 15.0 pg/mg), whereas the cortisol-21-HS EIA revealed an about 10-fold higher overestimation (193.15 ± 68.65 pg/mg) of the same sample set. Similar results were obtained for the marmots, where 21-HS EIA concentrations (60.6 ± 18.6 pg/mg, Table 3) did not reflect the hGC determined by LC-MS/MS at all, whereas the use of the 3-CMO EIA revealed three times higher values (2.03 ± 2.1 pg/mg LC-MS/MS vs 6.04 ± 1.92 pg/mg 3-CMO, Table 3). In the hyena, the mean 3-CMO EIA amount (13.58 ± 4.12 pg/mg) was just double, whereas the 21-HS EIA amount was 4-fold higher than the amount determined by LC-MS/MS (7.44 ± 2.24 pg/mg). In the black bear samples, almost no cortisone and cortisol were detectable by LC-MS/MS. This coincides with values obtained by the 3-CMO EIA that were the lowest for all examined species (Table 3), whereas the results obtained by 21-HS EIA were more than 10 times higher (50.8 ± 22.6 pg/mg) and thus were in the same range as those determined for lynx, hyenas and marmots with this assay (Fig. 2, Table 3).

With the limited samples available, we also performed a regression analysis between results obtained by 3-CMO EIA, 21-HS EIA and the LC-MS/MS concentration of the sum of cortisone + cortisol (Table 3). The results indicated for a significant relationship between LC-MS/MS and 3-CMO analysis in case of the mongoose, the lynx, the hyena and the marmot (Table 3), but only for the mongoose and the lynx a significant relation was found between LC-MS/MS and 21-HS EIA results. For the black bear, both EIA results were not correlated to the sum of cortisol + cortisone analyzed by LC-MS/MS. This might be related to the very low hGC concentration determined by LC-MS/MS.

HPLC analyses of immunoreactive GC in hair samples

To further elucidate the differences between both cortisol-EIA, we performed HPLC immunograms to characterize the immunoreactive compounds in hair extracts of six mammalian species (Fig. 3). For the six species analyzed, very different results were obtained, reflecting the specificity of both cortisol antibodies towards different compounds extracted from mammalian hairs. In all samples, the 3-CMO EIA

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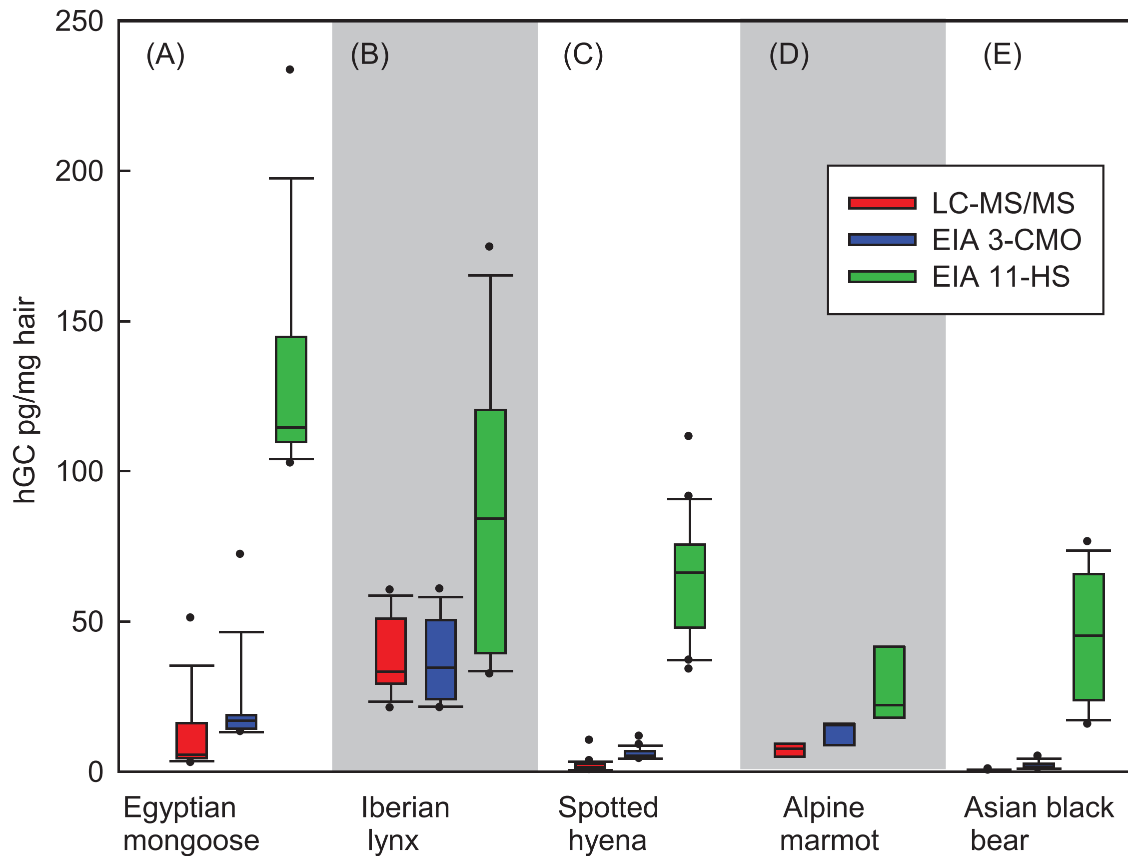

Figure 2: Box plots of hair cortisol content determined by LC-MS/MS (sum of cortisol and cortisone) and by two cortisol EIA, cortisol-3-CMO EIA and cortisol-21-HS EIA.

detects immunoreactive substances in Fraction 11 and, more pronounced, in Fraction 13–14, which coincide with elution peaks of cortisone and cortisol, respectively. The cortisol-21-HS antibody, however, has been found to be less specific, since it additionally cross-reacted with unknown compounds in Fractions 3–7, and depending on species, with substantial amounts of unpolar substances in fractions above 33.

In detail, in mongoose hair extracts (Fig. 3A) the proportions of cortisone and cortisol together represent only 10.5% of the total immunoreactivity when using the cortisol-21-HS EIA. Despite a major peak between Fractions 3–7, most immunoreactivity was found contributing to an unclear profile with peaks in Fractions 45, 50 and 54 not corresponding to any of our steroid standards. Moreover, some of them were less polar than our most unpolar standard in Fraction 45 (progesterone). Clearly different results were achieved with the cortisol-3-CMO assay, identifying cortisol (Fraction 13/14; 34.6%) and cortisone (Fraction 11; 10.9%) as the most prominent peaks. In addition, a polar peak in Fractions 3–6 has been found (11.8%), probably representing conjugated GCs. Following two minor peaks in Fractions 20 and 24, the profile nearly runs on baseline without the presence of questionable unpolar compounds. No significant peaks were detected on the position of corticosterone (Fraction 23). HPLC-immunograms from the lynx (Fig. 3B) clearly revealed two major peaks in Fractions 11 and 13 representing the elution positions of cortisone and cortisol, respectively, when using the cortisol-3-CMO assay, escorted by marginal amounts of additional compounds. For comparison, the cortisol-21-HS assay detects similar quantities of cortisone and cortisol; however, substantial amounts of additional less polar immunoreactivities were recognized in Fractions 18–22, 31, 33–35 and between 43–50. None of these immunoreactivities fits with the elution position of any of our standards. Altogether, the cortisol-3-CMO EIA specifically identifies cortisone and cortisol, both representing 67.4% of total immunoreactivity.

Similar deviating immunograms were found when checking both EIA in samples from the cheetah (Fig. 3C). As in the lynx the cortisol-21-HS EIA depicts considerable amounts of unknown compounds, whereas the use of the corresponding 3-CMO EIA increases the specificity towards cortisone + cortisol up to 65.4%.

A similar difference between both cortisol-EIA was found for hair samples from the spotted hyena (Fig. 3D). Again,

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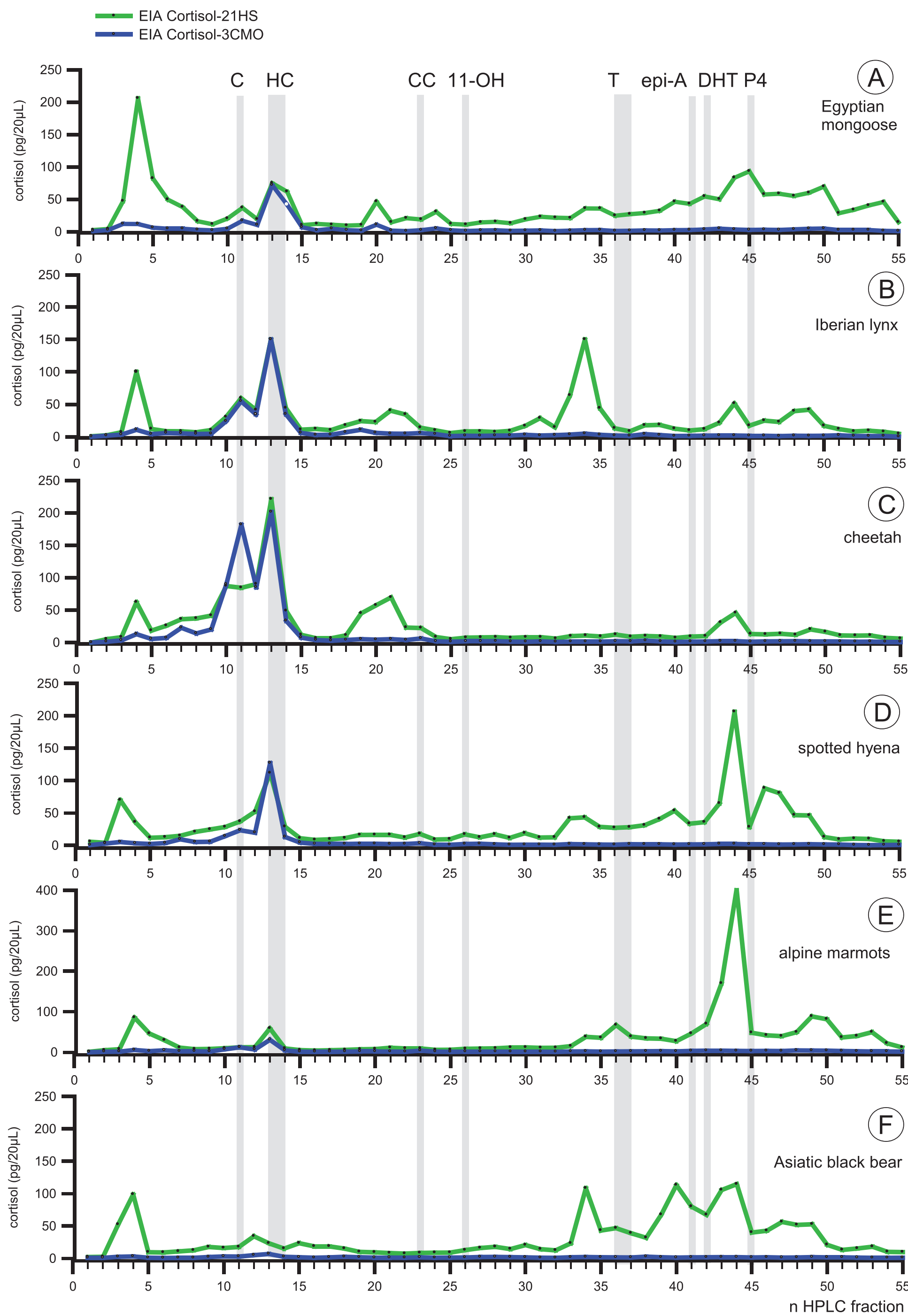

Figure 3: High-performance liquid chromatography (HPLC; reversed phase) separations of immunoreactive cortisol metabolites in pooled hair samples from six different mammalian species. The obtained fractions were analyzed with a cortisol-3-CMO EIA (blue lines) and a cortisol-21-HS EIA (green lines). The elution positions of reference standards are indicated by vertical lines; 11: C (cortisone); 13/14: HC (cortisol); 23: CC (corticosterone); 26: 11-OH (11-hydroxyetiocholanolone); 36/37: T (testosterone); 41: epi-A (epi-androsterone); 42: DHT (dihydrotestosterone); 45: P4 (progesterone). Pooled samples of A: Egyptian mongoose (*Herpestes ichneumon*, *n* = 294, 550 mg hair), B: Iberian lynx *(Lynx pardinus*, *n* = 93, 530 mg hair), C: cheetah (*Acinonyx jubatus*, *n* = 1, 900 mg hairs), D: spotted hyena (*Crocuta crocuta*, *n* = 7, 630 mg hair), E: marmot (*Marmota marmot*, *n* = 20; 900 mg hair) and F: Asiatic black bear (*Ursus thibetanus*, *n* = 14, 630 mg hair) were used.

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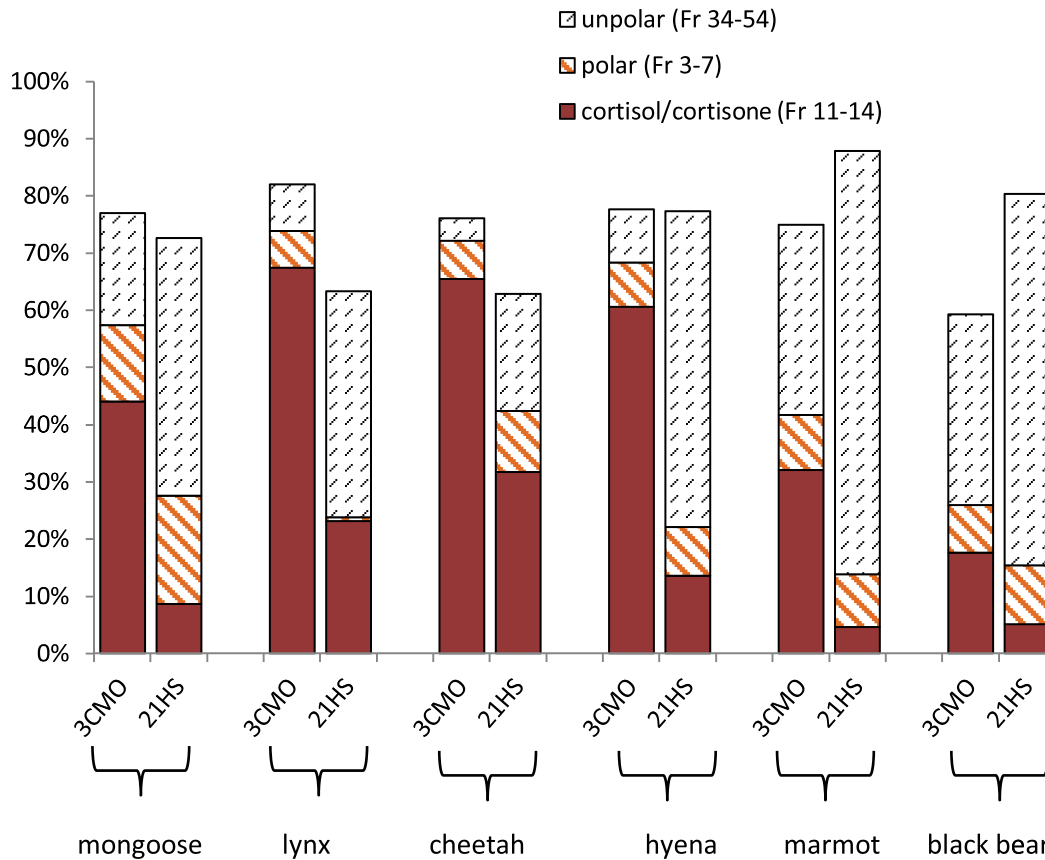

Figure 4: Comparison of HPLC immunograms from six mammal species showing the percentage of compounds contributing to the HPLC-immunograms. Fractions 3–7: polar, probably conjugated GCs; Fractions 11–14 cortisol and cortisone; Fractions 34–54: unpolar compounds.

the cortisol-21-HS assay cross-reacted with unpolar compounds eluting between Fractions 33 and 49 and peaking in Fraction 44 close to the elution position of progesterone. The cortisol-3-CMO assay only depicts cortisone and cortisol which together make up 60.6% of the total immunoreactivity.

In the marmot, the differences between both EIA were even more profound (Fig. 2E). From the total amount of immunoreactivity detected by the 21-HS assay, only a very small proportion (3.5%) was comprised of cortisol (Fraction 13) and probably cortisol conjugates (Fraction 4–6). Moreover, the 21-HS assay detected unpolar immunoreactivities with a peak in Fraction 44. Again, the 3-CMO assay was found to be more specific detecting cortisol and cortisone, which together represent 32.1% of the total immunoreactivity.

In the black bear (Fig. 3F), however, no distinct peak of immunoreactivity could be assigned to cortisone and cortisol, respectively, neither when testing the 21-HS assay nor the 3-CMO assay. This might be due to very low cortisol concentrations in hairs of black bears, as determined by LC-MS/MS (Table 3). The 21-HS assay cross-reacts with unknown compounds that would falsify the results. By contrast, it appeared that the 3-CMO assay detected small amounts of cortisol (the lowest of all species studied so far) which can be suggestively seen at the position of the cortisol standard (Fraction 13) apparently unaffected by other compounds.

Summary of all HPLC-immunograms: a species comparison

Based on the HPLC immunograms of the investigated species (Fig. 3A–F), the cortisol-3-CMO EIA was determined to be the assay of choice when compared with the corresponding 21-HS assay. An exception might be the black bear where the results were less clear. All results are summarized in Fig. 4, whereby Fractions 11–14 correspond to the sum of cortisone and cortisol, Fractions 3–7 to presumed polar conjugates of GC and Fractions 34–54 to compounds less polar than corticosterone found to be elevated in all HPLC immunograms causing falsification of measurements.

Except in the lynx, both assays detected the proportion of the most polar immunoreactivities in Fractions 3–7 in a

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similar order. Huge differences existed regarding the unpolar compounds between Fractions 34 and 54, with a distinctly smaller or absent affinity of the 3-CMO assay towards these compounds in all species, simultaneously improving the specificity of the 3-CMO assay towards cortisone and cortisol above 60% in the lynx, the cheetah and the hyena.

Discussion

Our comparative hGC analysis in six mammalian species demonstrate that results of cortisol EIA may lead to an overestimation of hGCs and that in dependence of chosen immune assay erroneous results might be obtained. The method of choice to exactly quantify hGC is LC-MS/MS (Russell *et al.*, 2015; Carlitz *et al.,* 2016). The LC-MS/MS technique recognizes the analyte by the combined actions of liquid chromatography separation and the specific composition of mass/charge ratio of fragments generated during mass spectrometry allowing the simultaneous quantification of cortisol, cortisone and corticosterone and their differentiation from putative precursors or metabolites and from other steroids, such as testosterone and progesterone. Unlike when using EIA, this high specificity prevents an impact of similar steroids or non-steroidal compounds that may bias the quantification of cortisol, cortisone and corticosterone. In addition, LC-MS/MS methods have shown good reproducibility and high specificity and can be used to analyze multiple steroids simultaneously (Parikh *et al.*, 2018).

Hair has been recognized as a biomaterial that accumulates GC hormones (Davenport *et al.,* 2006; Kirschbaum *et al.*, 2009; Macbeth *et al.,* 2010) and is supposed to reflect average blood serum level. In contrast to faeces, authentic hormones are present within the inert hair matrix as shown for human and many other species, although the precise mechanisms by which steroid hormones are incorporated into hair are still not fully understood (Ito *et al.,* 2005). In addition to systemic origin, a local synthesis of GC by the hair follicle is suggested (Cirillo and Prime, 2011) which may impact the steroid pattern in hairs. Our LC-MS/MS quantification of hGC in different mammalian species revealed different relations between cortisol, cortisone and corticosterone. For the hair of mongoose, hyena and marmots, cortisol is present in identical amounts compared to cortisone, whereas in the Iberian lynx and the cheetah cortisone is almost twice as high as cortisol. Corticosterone was found at very low levels. In the hair of Asiatic black bear, however, corticosterone was identified being the only GC measurable in this species (Table 3). Different serum levels as well as cortisol/corticosterone ratios were also described in different wildlife species (Koren *et al.*, 2012a), which were also unstable across individuals. In addition, diverging hair matrices may also contribute to species specific incorporation and storage patterns (e.g. impact of structural differences, different depositions of oils or pheromones and climatic conditions).

The relation between the different GC in blood serum might be an indicator for endocrine misbalances (Parikh *et al.,* 2018) or physiological stages like hibernation, and the cortisol/cortisone ratio in serum is not necessarily reflected in hGC. In human serum, the cortisol/cortisone ratio is about 5 : 1 (250 ng/ml cortisol vs. 50 ng/ml cortisone) (Eisenhofer *et al.*, 2017), whereas in hairs the relationship was found to be opposite with 7 pg cortisol vs. 25 pg cortisone per mg hair (1 : 3.5) (Stalder *et al.,* 2013). It is known that the cortisol/cortisone ratio is regulated by the enzymes 11β-HSD1 and 11β-HSD2. While 11β-HSD2 is responsible for producing cortisone from cortisol, 11β-HSD1 works predominantly in the opposite direction, reducing cortisone to cortisol in the liver, adipose tissue and other tissues (Liu *et al.*, 2019). Thus, the cortisol/cortisone ratio is not determined by GC production from the adrenal, but rather by the metabolic activity of target cells, including hair follicles.

Both EIA presented in this study were in-house made based on a polyclonal antiserum against cortisol, and both assays were also validated for non-invasive monitoring of fGMs. Those against cortisol-3-CMO have been previously utilized to non-invasively monitor changes in adrenal activity in faecal samples from hyenas (Benhaiem *et al.*, 2012); those against cortisol-21-HS were previously used for cortisol analyses in blood (Voigt *et al.*, 2004) and to non-invasively monitor adrenal activity in faeces from bats (Kelm *et al.,* 2016). Antibodies against cortisol-3-CMO are used quite commonly to monitor adrenal activity in several primate species such as Barbary macaques (*Macaca sylvanus*), lowland gorillas (*Gorilla gorilla*) and common marmosets (*Callithrix jacchus*) (Heistermann *et al.*, 2006), and in domestic dogs (*Canis lupus familiaris*) (Schatz *et al.*, 2001). Remarkably, both EIA were found unsuitable to monitor adrenal activity in the Iberian lynx based on fGM (Pribbenow *et al.*, 2014).

Faecal samples from carnivores usually contain only minor amounts of the original hormone (Young *et al.*, 2004); thus, it is not surprising that EIA directed towards the original steroids are unsuitable in some species. In hair extracts, however, we expect to find the non-metabolized hormones, thus cortisol assays should be able to detect cortisol accordingly. Our present data, however, demonstrate that cortisol-EIA based on different antibodies may lead to substantially deviating data, not only in respect of the hormone quantities determined but also providing misleading results. We analyzed the baseline variation in hair cortisol of Egyptian mongooses and highlight the importance of accounting for influences of age, sex and storage time by applying the 3-CMO EIA (Azevedo *et al.,* 2019). Determination of hGC with the 21-HS EIA was performed on the same sample set. When comparing both EIA (Table 1), higher putative amounts of GC were detected when using the cortisol-21-HS test. These results might lead to the assumption that cortisol-21-HS was superior compared to cortisol-3-CMO, but our HPLC immunograms revealed exactly the opposite. A pronounced cross-reactivity towards unknown substances extracted from hair was found to be very obvious, instead

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of a higher specificity toward hGC (Fig. 3). The results of both assays were significantly correlated (Fig. 1), but when we replicated the models used to the assess the effect of age, sex, season and sample storage time (Azevedo *et al*. 2019), the model using the 21-HS EIA data set did not show an effect of sex and storage time (Table 2, Supplement Table 3).

In the Iberian lynx, we achieved the best concordance between both EIA, with mean levels that are only 2.3 times higher when using the cortisol-21-HS assay (Table 1). Despite this apparently acceptable difference, the HPLC-immunogram showed very similar amounts of cortisol and cortisone (Fig. 3B) but also increasing amounts of unknown compounds between Fractions 33 and 55 with an additional peak in Fraction 34 in case of the cortisol-21-HS assay. Nevertheless, concentrations measured with both EIA do significantly correlate with each other. When applying the cortisol-3-CMO assay, however, the hGC concentrations almost exactly matched with the cortisol + cortisone amount determined by LC-MS/MS, verifying this EIA as more appropriate for hGC analyses in the lynx. In contrast, hair samples collected from Egyptian mongooses and marmots revealed 8- to 10-fold higher hGC concentrations when using the cortisol-21-HS EIA. In addition, comparative HPLC analysis (Fig. 3) confirmed that in these species cortisol-3-CMO was the more appropriate assay, even if hGC concentrations were still double to 3-fold compared to LC-MS/MS cortisol + cortisone amounts (Table 3, Fig. 2), still indicating an overestimation of hGC by this EIA. This agrees with data from Russell *et al*. (2015). They determined hGC in human hairs with four immunoassay methods, which were highly and positively correlated with two LC-MS/MS methods. However, LC-MS/MS hGC concentrations were between 2.5 and 50 times lower in comparison to the EIA results. The authors suggested employing a correction factor for each lab to achieve similar ranges of normal levels.

Our data on hair analysis in wildlife species contribute to the growing data base of studies that aim to use hGC (Koren *et al*., 2002; Davenport *et al.,* 2006; Accorsi *et al.,* 2008; Comin *et al*., 2011) to evaluate chronic stress, in most of which assays based on antibodies directed against cortisol have been applied (Koren *et al*., 2008; Ashley *et al.,* 2011; Carlitz *et al*., 2014; Peric *et al*., 2018). According to our results, different antibodies generated different immunograms indicating that besides cortisol and cortisone, unknown immunoreactive compounds can be extracted from hair in different quantities. Additionally, external factors on regions of growing hair may influence the composition of immunoreactive substances (Salaberger *et al.,* 2016). The comparison of HPLC immunograms established from sheep hairs exposed to dexamethasone fluid revealed significantly different compositions, when applying a cortisol-3-CMO and a cortisol-21-HS antibody, respectively.

It might be suggested that the method of steroid extraction influences the outcome of HPLC immunograms. Although we did not compare the two extraction methods for hair cortisol directly, our results indicate that irrespectively of the extraction method, high amounts of interfering unpolar components were determined with the cortisol-21-HS EIA. This applies to hairs from the mongoose and the lynx where hairs were milled and extracted for 30 min as well as from the other four species, where overnight extractions at 45°C were carried out. Independently of the extraction method, all HPLC immunograms were characterized by distinct amounts of interfering unpolar components between Fractions 34–54 (Fig. 3) when using the cortisol-HS-21 EIA, but obviously better results were achieved with the cortisol-3-CMO assay. Best results with more than 60% of immune reactivity contributing to cortisol + cortisone were obtained for hyena and the cheetah (no milling) and for the pooled milled Iberian lynx samples (Fig. 3).

The large amounts of unknown immunoreactivities between Fractions 34 and 54 might be caused by pooling and concentrating the extracts prior to HPLC-analyses, which uses extracts arisen from 500–1000 mg of hairs. Pooling is suggested to enhance inclusion of potential interfering compounds from the matrix (Koren *et al*., 2012b). Against this suggestion argues the parallelism of hair extracts in case of both EIA (data not shown), and the observation that all HPLC-immunograms showed comparable amounts of cortisone and cortisol at their corresponding elution positions (Fraction 11 and Fraction 13/14, respectively) when applying both cortisol EIA (Fig. 3). The unknown unpolar compounds, however, were exclusively detected by the cortisol-21-HS EIA. These unknown immunoreactivities lead to a substantial overestimation of hGC concentration when using the cortisol-21-HS EIA, which was up to 12-fold for African black bear (Table 1). Similar results were obtained by Kroshko *et al*. (2017) when quantifying hGC in grizzly bears applying different commercial ELISA kits commonly used for hGC determinations. Mean concentrations between 0.70 and 9.35 pg/mg of hairs were measured, resulting in a difference up to 13-fold between kits. Because nearly all commercial ELISA kits are originally designed for diagnostic use in plasma or serum, adapting them for quantification of hGC introduces potential for cross-reactions of unknown substances extracted from hair as shown in our comparison between the HPLC immunograms. Thus, absolute hGC values amongst studies of the same species that use different ELISAs should be viewed with caution.

As a result of our study, we decided to use the cortisol-3-CMO EIA for hGC analysis of mammalian hair extracts. In four of five tested species (mongoose, Iberian lynx, hyena, marmot), the determined amount of hGC nicely correlated with the LC-MS/MS determination of cortisol + cortisone, despite the overestimations in the mongoose, the hyena and the marmot (Table 3). This was not the case in the black bear where both EIA failed to produce reasonable results, possibly due to a very low content of hGC. The suitability of the 3-CMO EIA was demonstrated based on its strong specificity towards cortisone and cortisol, and low cross-reaction with other (unknown) mainly unpolar compounds

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extracted from hair. Distinct species differences not only in mean hGC concentration but also in the cortisol/cortisone ratio were demonstrated by both LC-MS/MS and HPLC immunograms.

Although we just used two different cortisol EIA, overall recommendation towards EIA selection for hGC analysis in wildlife can be deducted. In all investigated species, except the Asiatic black bear, we show that cortisone and cortisol were simultaneously present in hair extracts. Consequently, an appropriate EIA should cross-react to at least these two GC to account for any change of the cortisol/cortisone ratio in respect to physiological changes (Koren *et al.*, 2012a, Parikh *et al.*, 2018) or species-specific mechanisms of GC incorporation and storage within hair matrices. A selected antibody (EIA) should also express negligible affinity towards unknown substances that are less polar than corticosterone probably generating overestimations that could be misinterpreted as increased adrenocortical activity. Exemplarily for the mongoose we demonstrated that choosing the wrong EIA for hGC analyses lead to results that confound interpretation of adrenocortical activity. This validation can only performed by applying HPLC immunograms. Finally and most importantly, for any non-invasive stress related analysis, a proper pharmacological or biological validation is inevitable to demonstrate that data obtained are in relation to the adrenocortical activity (stress). Irrespective the source of hair GC, local or systemic, experiments in dairy cattle (Gonzalez-de-la-Vara *et al.*, 2011), goats (Endo *et al.*, 2018), Canada lynx (Terwissen *et al.*, 2013) and chipmunks (Mastromonaco *et al.*, 2014) showed that repeated ACTH treatments from 2 weeks to 2 months were sufficient to affect hGC. However, HPA activation by a single ACTH doses has been shown to be insufficient (Ashley *et al.*, 2011). This clearly supports hGC as a biomarker of repeated or even long-term stress events. The use of validated EIA or even the gold standard LC-MS/MS will generate more studies on wild animals and will also elucidate further species specific differences for this attractive non-invasive approach.

Supplementary material

Supplementary material is available at *Conservation Physiology* online.

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4. Article 3 – hair glucocorticoid analysis in the Iberian lynx

Azevedo A, Wauters J, Kirschbaum C, Serra R, Rivas A & Jewgenow K. (2020). Sex steroids and glucocorticoid ratios in Iberian lynx hair. *Conservation Physiology,* 8(1), coaa075. https://doi.org/10.1093/conphys/coaa075

The aim of this research was to validate the measurement of steroids (sex steroids and glucocorticoids) in Iberian lynx (*Lynx pardinus*) hair samples. First, we determined cortisol, cortisone, corticosterone, testosterone and progesterone in Iberian lynx hair using EIA. Secondly, we cross-validated the EIA by comparing it results to gold standard measurements obtained by high-pressure liquid chromatography coupled to tandem mass-spectrometry (HPLC-MS/MS) from the same extracts. We then compared the EIA immunoreactivities determined by HPLC with the elution positions of authentic steroids. Finally, we analysed the variations of steroid measurements according to Iberian lynx age, sex and origin, as well as behaviour and management variables.

Author contributions:

AA: conception and design, laboratory analysis and data acquisition, analysis and interpretation, writing, review and editing; JW: analysis and interpretation, writing, review and editing; CK: laboratory analysis data acquisition (LC-MS/MS), review and editing; RS: data acquisition, review and editing; AR: data acquisition, review and editing; KJ: conception and design, analysis and interpretation, review and editing, supervision.

Results of the study:

1. All of the measured steroids except corticosterone were detectable in Iberian lynx hair, with dehydroepiandrosterone being the most abundant.

2. EIA measurements of cortisol were overestimated due to antibody cross-reactivity with cortisone and were correlated to sum of the HPLC-MS/MS measurements for cortisol and cortisone. When measured by HPLC-MS/MS, cortisol was higher in females than in males, but the corresponding EIA results were confounded by the lack of antibody specificity.

3. Cortisol and cortisone appeared to show inverse trends according to sex and origin of Iberian lynx. Accordingly, cortisol-cortisone and cortisol-dehydroepiandrosterone ratios were more sensitive to glucocorticoid differences than the single hormones.

4. Longitudinal hGC measurements of an Iberian lynx that escaped after a wildfire showed an inversion of the cortisol-cortisone ratio that had subsided a year later. These results suggest that the measurement of several steroids such as cortisol, cortisone and DHEA can contribute to a better understanding of how hGCs reflect chronic stress responses.

5. Hair progesterone measured by EIA was overestimated by cross-reaction with 5α-dihydroprogesterone, a biologically active gestagene, and was highly correlated with HPLC-MS/MS results. Progesterone was higher in adult females compared to all other age-sex groups, indicating potential applications for non-invasive sex determination in Iberian lynx.

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SOCIETY FOR EXPERIMENTAL BIOLOGY
Research article

Sex steroids and glucocorticoid ratios in Iberian lynx hair

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Knowledge on species' reproductive biology is a fundamental pre-requisite of every conservation effort, but is often lacking. Sex steroids can provide valuable information for the assessment of reproductive success, whereas glucocorticoids are used to assess adrenocortical activity and stress-related bodily adaption. However, due to their perilous condition, access to animals is often difficult, which makes hormone measurement in non-invasively collected hair samples an attractive option. We determined cortisol, cortisone, corticosterone, testosterone and progesterone in Iberian lynx hair using enzyme immunoassay (EIA). Cross-validation was performed with high-performance liquid chromatography (HPLC) and high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS). Finally, we statistically evaluated the variations of sex steroids and glucocorticoids according to age, sex, origin, behavior and management. All steroids except corticosterone were detectable in Iberian lynx hair. Hair progesterone measured by EIA was overestimated by cross-reaction with 5α-dihydroprogesterone, a biologically active gestagene, and was highly correlated with HPLC-MS/MS results. Progesterone was higher in adult females compared to all other age-sex groups. Cortisol measured by EIA was overestimated due to antibody cross-reactivity with cortisone and was correlated to the sum of HPLC-MS/MS measurements for cortisol and cortisone. Cortisol was higher in females than in males measured by HPLC-MS/MS, but the EIA results were confounded by the lack of specificity. When using cortisol-cortisone and cortisol-dihydroepiandrosterone ratios, differences were noted between wild-caught and captive-bred lynxes. Additionally, longitudinal EIA measurements of an Iberian lynx after a wildfire showed an inversion of the cortisol-cortisone ratio that later subsided. These results validate the use of hair progesterone measurement for Iberian lynx reproductive monitoring and add to the growing evidence supporting the need for a more comprehensive approach to hair steroid measurement that accounts for local interconversion and co-regulation mechanisms.

Key words: Cortisol, cortisone, EIA, hair, HPLC, LC-MS/MS

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Introduction

Conserving and restoring populations of wild species has become a global necessity in the attempt to slow down current extinction rates (Hoffmann *et al*., 2010; Pimm *et al*., 2014). Common approaches involve interventions in the wild, coupled to captive breeding and management of 'rescue net' populations for reintroduction (Ebenhard, 1995; Mallinson, 1995). In many cases, the biology of the targeted species is poorly known, and knowledge needs to be gathered as part of the initial establishment of conservation programs. Questions related to species' reproductive biology and physiology need to be answered promptly in order to ensure health, survival and reproduction in both captive and wild populations. Unfortunately, for many species this information is still lacking (Wildt *et al*., 2010). Measurement of steroid sex hormones provides a valuable tool for understanding the reproductive biology and ecology of wild species, thereby supporting development of assisted reproductive techniques (ARTs) (Wildt and Wemmer, 1999; Schwarzenberger, 2007; Comizzoli *et al*., 2009; Jewgenow *et al*., 2017). Similarly, the measurement of glucocorticoids has become accepted for the assessment of behavior, stress and allostatic load in captive and wild populations (Möstl and Palme, 2002; Sheriff *et al*., 2011; MacDougall-Shackleton *et al*., 2019). Steroid hormone measurements have traditionally been performed using enzyme immunoassays (EIAs), which remain the most viable option for wide-scale daily application in field conservation, despite the availability of more specific yet more expensive and skill-demanding techniques such as (ultra) high-performance liquid chromatography ((U)HPLC) facultatively coupled to liquid chromatography-mass spectrometry (LC-MS).

Animal populations under conservation management are often in perilous conditions, with very few specimens remaining and low genetic diversity. Because of the risks involved in capture, sampling procedures should be reduced to the minimum, complicating the use of matrices that need to be collected invasively and/or repeatedly, like blood serum or plasma. These limitations have been circumvented by measuring hormones and their metabolites in feces, urine and hair (Sheriff *et al*., 2011). Hair is thought to reflect the longest time period of circulating hormone levels in a single measurement, is easy to store for long periods and does not require special shipping conditions. It has unsurprisingly become popular for stress research in wildlife species over the past 2 decades (Meyer and Novak, 2012; Russell *et al*., 2012; Stalder and Kirschbaum, 2012). In the field of reproductive biology, measurement of hormones in hair has not been as popular, with validation studies performed in only a few species of wildlife (Koren *et al*., 2019).

Several authors have alerted to the uncertainties that should be addressed before applying EIA to measure steroids and their metabolites in feces (Touma and Palme, 2005; Pribbenow *et al*., 2017; Palme, 2019) and in hair (Koren *et al*., 2019). Currently, uncertainties persist on how hormones are incorporated into the hair shaft and how much local production of steroids influences the results (Ito *et al*., 2004; Slominski *et al*., 2007; Sharpley *et al*., 2009; Keckeis *et al*., 2012; Salaberger *et al*., 2016). Furthermore, assays used without validation can confound steroid measurements due to cross-reaction with unintended compounds (Jewgenow *et al*., 2020). Additionally, biological validation is necessary to understand which time period of circulating steroid levels is reflected in hair samples (Kalliokoski *et al*., 2019). Nevertheless, based on the response to adrenocorticotropic hormone (ACTH) stimulation tests and correlation with the values of fecal, blood and salivary glucocorticoids, it is currently well established that hair glucocorticoids (hGCs) reflect adrenocortical activity (Kalliokoski *et al*., 2019). However, while hormonal variations caused by gender or reproductive cycles provide opportunities for validation, the number of studies focusing on sex steroid measurement in hair is so far limited in wild (Koren *et al*., 2006; Terwissen *et al*., 2014; Bryan *et al*., 2015, 2014; Cattet *et al*., 2017) and domestic species (Gleixner and Meyer, 1997; Terwissen *et al*., 2014; Tallo-Parra et al., 2018; Bergamin *et al*., 2019).

At the turn of the century, the Iberian lynx (*Lynx pardinus*) was the most endangered felid on Earth, with less than 200 animals remaining (Guzmán *et al*., 2004). Extensive conservation efforts have led to a population recovery over the past 2 decades, allowing the species to be downgraded from 'critically endangered' to 'endangered' by the International Union for Conservation of Nature (IUCN). Despite the successful genetic management of captive and wild populations (Kleinman-Ruiz *et al*., 2019), it remains threatened by the lowest genome-wide genetic diversity reported so far in any species (Abascal *et al*., 2016). Wild felids often have poor reproductive success in captivity (Andrews *et al*., 2019). Abortions, premature birth and infant mortality have been challenging problems in the Iberian lynx captive breeding program, leaving ART as a last resort to salvage the genetic diversity of founders that did not reproduce within their lifetime (Jewgenow *et al*., 2017). These techniques are also expected to play a role in improving meta-population management, by allowing gene flow between the unconnected populations without the movement of animals (Swanson, 2006). Consequently, cost-effective and non-invasive tools for monitoring the reproductive cycles hold great potential for pinpointing the adequate timing of reproductive management interventions and for assessing reproductive success, both in captivity and in the wild. Similarly, the non-invasive measurement of glucocorticoids offers a promising tool for the measurement of chronic stress in the captive Iberian lynx population or of reintroduced animals adapting to the wild. Additionally, glucocorticoid levels may serve as an indicator of allostatic load in free-ranging populations. In this study, we therefore evaluated the potential of cortisol, cortisone, corticosterone, testosterone and progesterone determination in Iberian lynx hair using EIA. Each assay was validated through identification of the immuno-reactive components in the hair extracts after HPLC separation. Additional validation

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regarding the specificity of the EIA results was pursued in a subset of samples applying gold standard high-performance liquid chromatography coupled to mass spectrometry (HPLC-MS/MS). Once methods were validated, we evaluated the variations in concentrations of sex steroids, glucocorticoids and selected hormone ratios according to age, sex, origin and behavior and management variables. Finally, we presented an example of longitudinal variation of hGC measurements in an Iberian lynx after an escape-recapture event caused by a wildfire.

Materials and methods

All chemical reagents were purchased from Sigma–Aldrich (Taufkirchen, Germany) unless stated otherwise and were of the highest purity available.

Sample collection

Samples from Iberian lynx were obtained during routine health checks within the Iberian lynx conservation breeding program in Spain and Portugal. While all animals were held in captivity, some had been born in the captive-breeding program (captive-bred) while others had been brought into the breeding program from the wild as founders (wild-caught). Animals were captured for health checks with cage traps that they entered voluntarily after a period of habituation. Hair samples were collected opportunistically from the standard sites where hair was clipped for venipuncture while the animals underwent routine health checks under anesthesia. If not indicated otherwise, the hair was clipped from the inner surface of hind legs. For the comparison of hormone levels in proximal and distal segments of hair samples, hair was clipped first at 0.5 cm length to obtain the distal segment, and the remainder was clipped as close to the skin as possible to obtain the proximal segment of 0.5 cm length.

Steroid extraction from hair

Previously, two methods were reported for the extraction of steroids in hair for glucocorticoid analysis (Jewgenow *et al.*, 2020). The first method describes extracts for glucocorticoid quantification by immunoassays (pulverized method), while the second method was adapted from Carlitz *et al.* (2016) and characterized by the extraction of whole hair (whole hair method) prior to glucocorticoid identification by HPLC-MS/MS. We screened the applicability of both methods for steroid (glucocorticoids, progesterone and testosterone) analysis in lynx hair by monitoring the repeatability of the extraction after quantification by EIA. In a first experiment, we examined the repeatability of glucocorticoid, progesterone and testosterone quantification, expressed by the coefficient of variation (CV, %), after comparing pulverized and whole hair extractions in five male and five female individuals, with two replicates per method for each individual.

Based on the results, and taking into account the complexity of the lynx hair samples, the experiment was repeated after including an additional sample preparation step for both methods, which comprised cutting the available hairs in 5 mm pieces followed by mixing prior to weighing of 20 mg hair fragments for further extraction. For the whole hair extraction, the method was adapted to Mastromonaco *et al.* (2014), an established method for steroid extraction for hair in wildlife. The main differences compared to the original method were the fraction of MeOH (80% versus 100%), the shaking time (24 h versus 18 h) and the shaking temperature (room temperature compared to 45 C). In this follow-up experiment, hair subsamples were subjected to both extraction methods with duplicate analysis included for the adapted whole hair method (three males and three females) and triplicate analysis for the pulverized hair method (two males and three females). The final extraction method selected for sample processing is described below.

Hair was cut into 5 mm pieces, mixed and an aliquot of 20 mg separated and washed twice with 2 ml of 90% methanol by vortexing for 5–10 s to remove surface contamination. Thereafter, the samples were dried for 1 h at 70 C and aliquots (~10 mg) from selected Iberian lynx ($n = 93$) were taken and milled to a fine powder with ceramic beads in a tissue homogenizer as described before (Azevedo *et al.*, 2019). Then 400 μl of 90% methanol were added to the powder and shaken at room temperature for 30 min. Following centrifugation (3 min, 1000 × g), the supernatant was collected and transferred into a new tube, diluted 1:2 with water and kept frozen until EIA analysis and the preparation of HPLC immunograms. From hair samples of 12 lynxes, additional extracts were produced for HPLC-MS/MS analyses (see below) according to the same protocol.

Steroid analyses by EIA

An in-house cortisol immunoassay was used based on polyclonal antibodies (rabbit) directed against cortisol-3-CMO-BSA and the corresponding 3-CMO-peroxidases label as previously described (Ludwig *et al.*, 2013). The antibody cross-reactivities of the cortisol-3-CMO assay were as follows: cortisol, 100%; cortisone, 19.5%; corticosterone, 6.3%; desoxycorticosterone, 0.1%; progesterone, 0.1%; estradiol, 0.1%; testosterone, 0.1%.

Cortisone was determined by a commercial EIA (Arbor Assays DetectX® Kit K017, Arbor Assays, An Arbor, USA). To comply with the manufacturer's guidelines, a 75–150 μl aliquot of each sample extract was freeze dried and resolved in 150 μl assay buffer. The EIA measurements were performed in duplicates as indicated for the kit. According to the providers, the cross-reactivities were as follows: cortisone, 100%; 5α-dihydrocortisone, 31.7%; prednisone, 9.0%; 5ß-dihydrocortisone, 4.4%; 11-dehydrocorticosterone, 0.62%; 20α-dihydrocortisone, 0.26%; < 0.1% for 11α-hydroxycorticosterone, 20ß-dihydrocortisone, corticosterone, cortisol, dexamethasone, estradiol and progesterone.

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Progesterone (P4) analyses were carried out with an in-house microtitre plate EIA as described earlier (Dehnhard *et al.*, 2008) using a commercial P4 antibody (Sigma P1922, raised in rats to progesterone) and 4-pregnen-3,20-dione-3-CMO-peroxidase label. The cross-reactivities to other steroids were as follows: 4-pregnen-3,20-dione (progesterone), 100%; 5α-pregnan-3,20-dione (5α-DHP), 76.8%; 5α-pregnan-3ß-ol-20-one (5a), 64.2%; 5-pregnen-3ß-ol-20-one, 12%; 4-pregnen-3αol-20-one, 4.2%; < 0.1% for 5ß-pregnan-3α,20αdiol, 4-pregnen-20α-ol-3-one, 5ß-pregnan-3α-ol-20-one, 5a-pregnan-20α-ol-3-one, 5α-pregnan-3α,20α-diol, 5a-pregnan-3ß,20α-diol, testosterone, estradiol and cortisol.

Testosterone was analyzed with an in-house EIA, using polyclonal antibodies (rabbit) against testosterone-3-CMO-BSA and the corresponding 3-CMO-peroxidase as label. The cross-reactivities were as follows: testosterone, 100%; dihydrotestosterone (DHT), 53.85%; 4-androsten-3ß, 17ß-diol, 4.6%; 19-nortestosterone, 2.3%; < 0,01% for 11ß-hydroxyetiocholanolone, 11-oxo-etiocholanolone, cortisol, corticosterone, 5α-androstan-17-one, androstendione, androsterone, 5α-androsterone, dihydroepiandrosterone (DHEA), testosterone-glucoronide and epiandrosterone.

The principle of the in-house EIA procedure has been described in detail (Finkenwirth *et al.*, 2010). All EIA measurements were conducted in duplicate and results were expressed as pg/mg hair weight. Serial dilutions of hair extracts showed parallelism to the standard, with no significant difference in slopes ($P > 0.05$). The inter- and intra-assay coefficients of variation (CV) for the EIA as well as the (linear) detection range are presented in Table S1 of the Supplementary Materials.

High-performance liquid chromatography coupled to mass spectrometry

Hair extracts from 12 selected Iberian lynxes (six males, six females) were adjusted to an absolute content of about 150 pg cortisol. To achieve this, cortisol concentration of extracts were determined by cortisol-3-CMO-EIA, and the required volume of each sample was determined according to the mean cortisol concentration (see Supplementary Table S2). For Iberian lynx, 200 μl (∼5 mg hair) of each extract were transferred to 1.5 ml reaction tubes, dried down for 90 min at 60 C under a constant stream of nitrogen and dissolved in 225 μl 50% methanol (LC-MS grade). The samples were sent to the Faculty of Psychology, Technische Universität Dresden, Germany, for HPLC-MS/MS analyses, based on a Shimadzu LC-20AD HPLC unit, a Shimadzu SIL-20AC autosampler and a Shimadzu CTO-20AC column temperature oven (Shimadzu, Canby, OR, USA) coupled to an AB Sciex API 5000 Turbo-ion-spray® triple quadrupole tandem mass spectrometer equipped with spheric pressure chemical ionization (APCI). Details on the liquid chromatography methodology and mass spectrometric conditions are described in Carlitz *et al.* (2016) and Gao *et al.* (2013).

High-performance liquid chromatography

HPLC was performed as described before (Jewgenow *et al.*, 2020) and aimed to characterize the compounds detected by the respective EIA. From the hair extracts of 93 lynxes, aliquots of 460 μl were taken corresponding to ∼530 mg of hairs and pooled before purification on Octadecyl C18 columns (0.5 ml, J.T. Baker, BAKERBOND SPE™ 7020-01) as previously described (Azevedo *et al.*, 2019). Eluates were evaporated in a heater at 55 C under nitrogen and dissolved in 200 μl of 40% methanol. About 130–150 μl of the re-suspended volume was injected on a reverse-phase Ultrasep ES100/RP – 18/6 μm HPLC column (4 × 250 mm, Sepserv, Berlin) and separation was achieved by using a methanol + water mixture with the following gradient: 60% methanol during 5 min, 60–90% methanol during 10 min, 90–100% methanol during another 10 min. The flow rate was 1 ml/min. Fractions of 0.33 ml were collected at 20 s intervals over a period of 25 min. All fractions were lyophilized and re-suspended in 200 μl 40% methanol before 20 μl aliquots were analyzed by EIA. The elution positions of native cortisone, cortisol, corticosterone, 11-hydroxyetiocholanolone, testosterone (T), progesterone (P4), 5α-pregnan-3,20-dione (5α-DHP) and 5α -pregnan-3α,20α-diol (5a) on this column had been previously determined in separate HPLC runs.

Statistical methods

All statistical analyses were conducted in R version 3.5.1 (R Core Team, 2017). The association between paired values of hormone EIA measurements from proximal and distal segments of hair samples was tested using Pearson's correlation, following the assessment of scatterplots for linear covariation and Shapiro–Wilk normality tests and quantile–quantile plots for normality. In the single case of non-normality (cortisone), Spearman's rank-based correlation was used. For comparisons of the variation of each hormone EIA according to age class (animals between 6 months and 2 years of age were considered juveniles, while animals older than 2 years of age were considered adults) and sex, a two-way analysis of variance with type III sum of squares for unbalanced sample sizes was conducted using the EIA results as dependent variables and age, sex and the interaction between age and sex as independent variables. Model residuals were checked for normality and homoscedasticity by visual inspection of residual plots and Shapiro–Wilk normality tests. Homogeneity of variances was checked by visual inspection of plots and Levene's test. *Post hoc* between-group tests were performed with Tukey–Kramer adjusted comparisons, using the multcomp package (Hothorn *et al.*, 2008). For the cortisol-cortisone ratio (Sollberger and Ehlert, 2016), a Kruskal–Wallis non-parametric test using age-sex group as the independent variable was used, followed by Dunn's *post hoc* test with Bonferroni correction, using the dunn.test package (Dinno, 2017). Mann–Whitney U-tests were used for the comparison of glucocorticoid EIA values and the cortisol-cortisone ratio (Sollberger and Ehlert, 2016) between captive-bred and wild-caught lynxes. For an

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analysis of the variation of hGC levels measured by cortisol-3CMO-EIA in relation to behavioral (mean frequency of repetitive behavior in the last month and the last year) and management variables (breeding center, enclosure area, days since last capture, number of lifetime captures, days since last transfer between institutions, number of lifetime transfers between institutions, days since last enclosure change and number of lifetime enclosure changes) available for a subset of females ($n = 19$), non-parametric Kruskal–Wallis, Mann–Whitney U-tests and Spearman's rank correlation tests were used. Comparisons of HPLC-MS/MS hormone measurements, and their ratios, between Iberian lynxes of different age, sex and origin (wild caught versus captive bred) were performed using non-parametric tests due to the small sample size ($n = 12$) and the non-normal distribution of complex variables such as ratios (Sollberger and Ehlert, 2016). Mann–Whitney U-tests were used for sex and origin, and Spearman's rank correlation was used for age.

Results

Steroid extraction from hair

Both the pulverized and whole hair methods initially showed a poor repeatability of steroid extraction from hair samples, significantly underperforming compared to the generally accepted limits of up to 15% (20% for small levels) of variation between duplicate or triplicate extractions from the same hair sample. Consequently, an extra homogenizing step that consisted in cutting and mixing hair samples prior to sample weighing and extraction was included in the protocol for both methods. This extra preparatory step improved the repeatability to within the acceptable limits (Supplementary Table S3) and was therefore included in our standard protocol for all subsequent analyses.

No significant concentration differences were observed between the pulverized and whole hair methods for all steroids in combined data of males and females. Nevertheless, the pulverized hair method consistently resulted in higher concentrations for progesterone in males, and in both males and females for testosterone, when compared to the whole hair method. Measured steroid concentrations with both the pulverized and whole hair methods, including gender comparison, are presented in the Supplementary Materials (Table S3).

Due to the best observed repeatability in the samples treated with the pulverized hair method, particularly for progesterone, this method was selected for extraction of the study samples.

Determination of steroid content in hair samples using EIA

All of the targeted steroids were measureable in Iberian lynx hair samples using EIA (Supplementary Table S4). With the exception of testosterone, all exhibited differences when comparing different age and sex groups (Figs 1 and 2). In the case of the cortisol-3CMO-EIA, sex had a significant effect on hGC measurements ($F_{(1, 33)} = 4.474$, $P = 0.004$). *Post hoc* Tukey–Kramer pairwise comparisons showed that juvenile females had lower hGC levels measured by the cortisol-3CMO-EIA than adult males ($\Delta = -11.8$ pg/g, 95% CI [−1.49, −11.81], $P = 0.02$), as did juvenile males ($\Delta = -13.4$ pg/g, 95% CI [−2.83, −24.06], $P = 0.01$). The values measured with the commercial cortisone EIA were significantly influenced by sex ($F_{(1, 25)} = 8.499$, $P = 0.007$) and the interaction between age and sex ($F_{(1, 25)} = 5.446$, $P = 0.027$). *Post hoc* pairwise comparisons revealed higher cortisone measurements in adult males compared to adult females ($\Delta = 15.6$ pg/mg, 95% CI [0.88, 30.35], $P = 0.03$), juvenile females ($\Delta = 21.4$ pg/g, 95% CI [6.38, 36.49], $P < 0.001$) and juvenile males ($\Delta = -22.9$ pg/g, 95% CI [−7.40, −38.33], $P = 0.002$). For progesterone EIA measurements, there were statistically significant effects of the age-sex interaction ($F_{(1, 33)} = 81.66$, $P < 0.001$), as well as age ($F_{(1, 33)} = 220.07$, $P < 0.001$) and sex ($F_{(1, 33)} = 161.82$, $P < 0.001$), with females presenting higher progesterone levels than males ($\Delta = -67.0$ pg/mg, 95% CI [−81.36, −52.83], $P < 0.001$), juvenile males ($\Delta = -73.1$ pg/mg, 95% CI [−87.34, −58.80], $P < 0.001$) and juvenile females ($\Delta = -75.6$ pg/mg, 95% CI [−89.38, −61.81], $P < 0.001$). A significant effect of age ($F_{(1, 33)} = 6.6485$, $P = 0.015$) on testosterone-EIA measurements was present, but *post hoc* pairwise comparisons revealed no significant differences between groups. When using the calculated cortisol-cortisone ratio, the results showed significant differences between age-sex groups ($\chi 2 = 11.90$, df = 3, $P = 0.01$). *Post hoc* Dunn's test revealed significant differences between adult and juvenile males ($P = 0.007$) and adult males and juvenile females ($P = 0.023$).

No significant difference was observed between EIA measurements of cortisol, cortisone and cortisol-cortisone ratio between wild-caught ($n = 7$) and captive-bred ($n = 30$) lynxes. Similarly, no effect of management and behavioral variables was found on hGCs measured by cortisol-3CMO-EIA, in the subset of females ($n = 19$) where these data were available (Supplementary Table S5). When comparing the EIA measurements between proximal and distal segments of the hair shafts (five males, five females), we found significant positive correlations for all the steroid hormones measured (Supplementary Table S6). The longitudinal follow-up of hGC levels in an Iberian lynx female following a 24-day escape-recapture event caused by a massive wildfire showed an increase in glucocorticoid levels measured by the cortisol-3CMO-EIA and a decrease in cortisone-EIA measurements ~3 weeks after recapture, resulting in an inversion in cortisol-cortisone ratio in relation to the recapture date (see Fig. 3). One year after recapture, cortisone-EIA measurements had increased and cortisol-3CMO-EIA measurements had decreased, thus reversing the inversion in cortisol-cortisone ratio that occurred in the 3 weeks post recapture.

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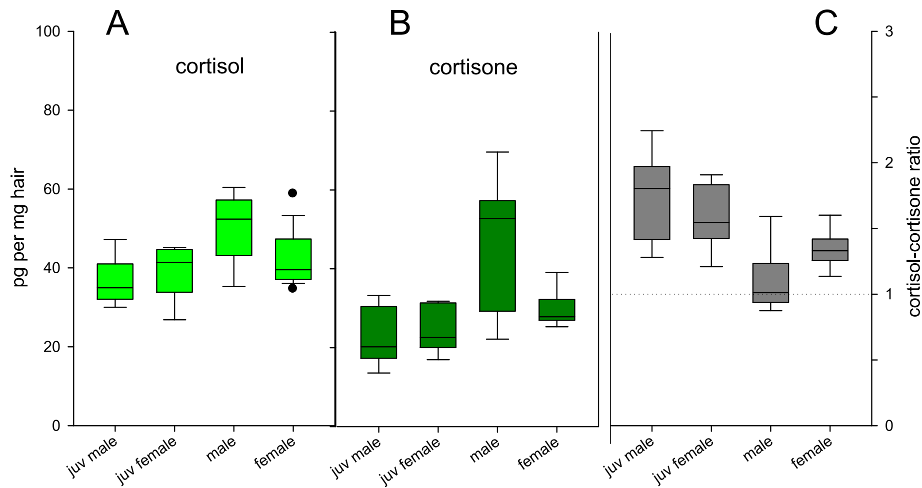

Figure 1: Iberian lynx hGCs (EIA) by age-sex group. Measurements of cortisol-3CMO-EIA (**A**) and cortisone-EIA (**B**) in pg/mg and calculated cortisol-to-cortisone ratio (**C**) based on EIA results, of hair from juvenile ($n = 9$) and adult male ($n = 10$), as well as juvenile ($n = 9$) and adult female ($n = 13$) Iberian lynxes

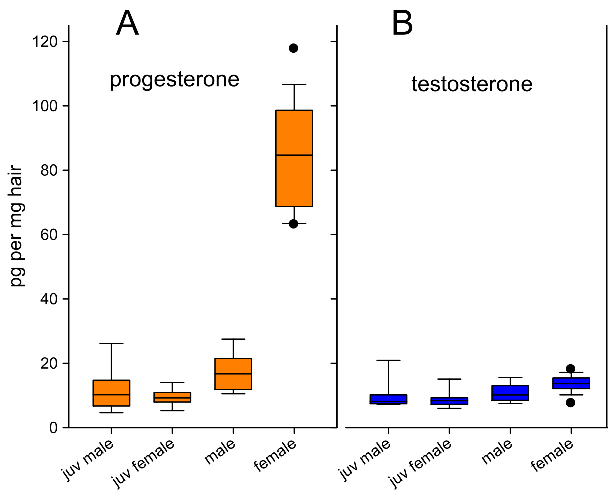

Figure 2: Sex steroids in Iberian lynx hair (EIA) by age-sex group. Measurements of progesterone-3CMO-EIA (**A**) and testosterone-3CMO-EIA (**B**) in pg/mg in hair from juvenile ($n = 9$) and adult male ($n = 10$), as well as juvenile ($n = 9$) and adult female ($n = 13$) Iberian lynxes

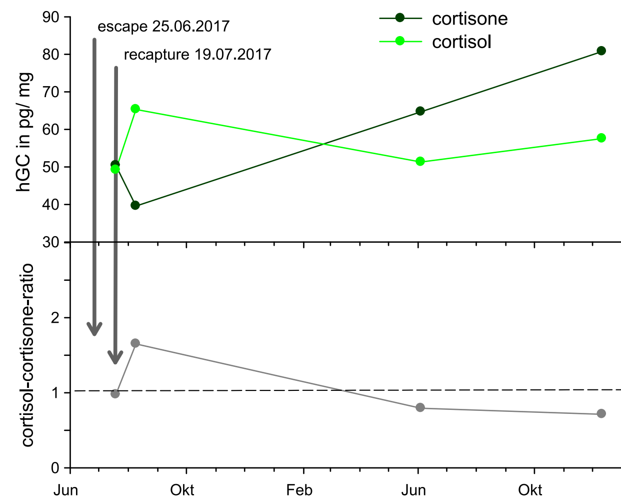

Figure 3: Inversion of cortisol-cortisone ratio in an Iberian lynx after a wildfire. Variation in cortisol-3CMO-EIA and cortisone-EIA measurements and their ratio in hair samples from an Iberian lynx named Fran, following a 24-day escape-recapture event caused by a massive wildfire that overcame the El Acebuche breeding center in 2017. Approximately 3 weeks after recapture, the relation between cortisol and cortisone had inverted, and 1 year later the inversion had been reversed

HPLC analyses of immunoreactive steroids

The HPLC immunograms (Fig. 4) show that the cortisol-3-CMO EIA reacted to both cortisol (fraction 13/14; 36%) and cortisone (fraction 10/11; 30%) with an overall immunoreactivity of 66%. Only marginal amounts of reactivity were detected throughout the remaining immunogram, indicating the EIAs specificity for these two hair glucocorticoids (therefore referred to as hGC). An even more specific HPLC-immunogram was obtained with the cortisone EIA, with only two peaks appearing at positions 10/11 (84%) and 13/14 (11%), which correspond to a total of 95% reactivity on a mostly non-reactive baseline.

In the case of the corticosterone-3-CMO-EIA, HPLC failed to detect any immunoreactivity in the positions corresponding to corticosterone (fractions 23). Peaks of cross-reactivity with unknown compounds were detected in fraction 4. The results are coincident with the very low or undetectable concentra-

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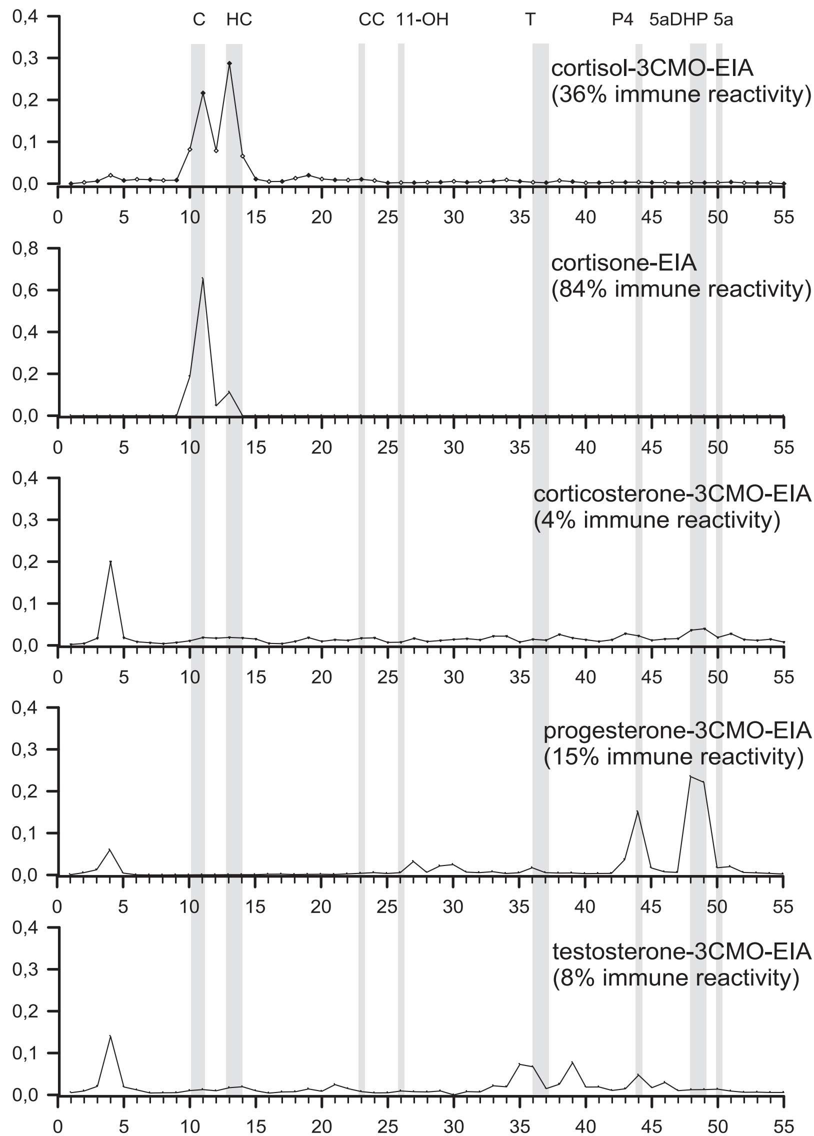

Figure 4: HPLC immunograms for EIA targeting steroid hormones in Iberian lynx hair. High performance liquid chromatography (reversed phase) separations of immunoreactive cortisol and progesterone metabolites in pooled hair samples from Iberian lynx *(Lynx pardinus*, $n = 182$, 900 mg hair). The obtained fractions were analyzed with a cortisol-3-CMO-EIA, a commercial cortisone-EIA, a corticosterone-3-CMO-EIA, a progesterone-3CMO-EIA and a testosterone-3CMO-EIA. The elution positions of reference standards are indicated by vertical lines, 10/11: C (cortisone), 13/14: HC (cortisol), 23: CC (corticosterone), 26: 11-OH (11-hydroxyetiocholanolone), 36/37: T (testosterone), 44–45: P4 (progesterone), 48–49: 5α-DHP (5α-pregnane-3,20-dione) 50: 5a (5α-pregnan-3ß-ol-20-one)

tions of testosterone and corticosterone, respectively, in the HPLC-MS/MS results for Iberian lynx hair.

In the case of the progesterone-3CMO-EIA, two peaks were identified in positions 44 and 48/49, corresponding to the elution positions of progesterone (15%) and 5α-pregnan-3,20-dione (5α-DHP; 46%), respectively. Other than a small 5% peak in fraction 3, there is little presence of cross-reactivity with known and unknown compounds, revealing that this EIA is adequate for the measurement of gestagens in Iberian lynx hair samples. The immunogram for the testosterone-3CMO-EIA revealed only 8% immunoreactivity in the position corresponding to testosterone (fraction 38).

Determination of steroid content in hair samples using HPLC-MS/MS

We performed quantitative HPLC-MS/MS determinations of cortisol, cortisone, progesterone, testosterone and DHEA in

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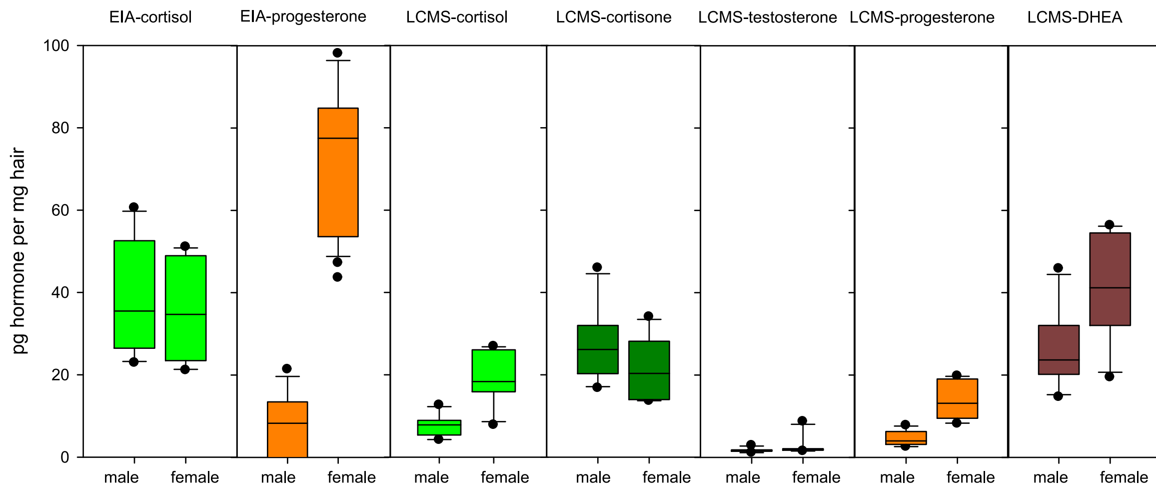

Figure 5: Steroid content in Iberian lynx hair measured by HPLC-MS/MS. Box-plots of steroid content in 12 Iberian lynx (six males, six females) determined by UHPLC-MS/MS. Values determined by cortisol-3-CMO-EIA and progesterone-3CMO-EIA in the same 12 samples were included for comparison. Sex differences are seen for progesterone and cortisol content in HPLC-MS/MS results. The difference between males and females appears overestimated in the progesterone-3CMO-EIA and attenuated in the cortisol-3CMO-EIA

a subset of 12 Iberian lynx samples (six males, six females) [LOD (limit of detection) = 0.32 pg/mg, LOQ (limit of quantification) = 1.05 pg/mg]. Cortisol and progesterone contents in these samples were also determined by the respective EIA to allow comparison (Fig. 5, Supplementary Table S7). The steroid recovered in highest concentration was DHEA, followed by cortisone and then cortisol. Sex steroids progesterone and testosterone were detected in lower concentrations, while corticosterone was not detectable.

The variation of HPLC-MS/MS steroid measurements and cortisol-to-cortisone and cortisol-to-DHEA ratios was analyzed according to sex (six female, six male), age (2 to 12 years) and origin (six wild caught, six captive bred). When comparing the results between sexes (Fig. 5), females presented significantly higher concentrations of progesterone and cortisol. The latter difference was also significant when evaluating sex differences in the cortisol-to-cortisone, but not the cortisol-to-DHEA ratio. No relation was found between age and any of the steroids measured. Comparison of HPLC-MS/MS measurements according to origin revealed a trend towards higher cortisol in wild-caught animals. This difference became highly significant when using cortisol-cortisone and cortisol-DHEA ratios (Fig. 5, Table 1).

Relation between HPLC-MS/MS and EIA measurements

We performed regression analysis between the results of cortisol-3CMO-EIA and progesterone-3CMO-EIA and the HPLC-MS/MS results for the same 12 hair samples. The analysis indicated a significant relation between the values obtained with the cortisol-3CMO-EIA and the HPLC-MS/MS values of cortisone ($r = 0.84$, $P < 0.001$) and cortisol + cortisone (named hGC) ($r = 0.86$, $P < 0.001$), but not with cortisol alone. In the case of the progesterone-3CMO-EIA, the results showed a significant correlation with the progesterone values measured by HPLC-MS/MS ($r = 0.86$, $P = 0.018$).

Discussion

In this study, we analyzed Iberian lynx hair samples for cortisol, cortisone, corticosterone, DHEA, progesterone and testosterone, using a combination of EIA, HPLC and HPLC-MS/MS to validate a group of assays that are applicable in field conservation. Our analysis shows that besides cortisol, cortisone, DHEA, progesterone and testosterone are detectable in Iberian lynx hair. DHEA was the most abundant steroid, followed by cortisone, cortisol, progesterone and testosterone, respectively. Corticosterone was not detectable.

Sex steroids such as progesterone and testosterone are important indicators for Iberian lynx conservation due to their potential role in pregnancy diagnosis and fertility assessment, respectively. The lynx genus is a unique case among felids due to the persistence of *corpora lutea* and elevated systemic progesterone levels beyond the duration of pregnancy (Jewgenow *et al.*, 2014). Additionally, variations in sex hormone concentrations in hair have been associated with social and nutritional stress in wolves (*Canis lupus*) and brown and black bears (*Ursus arctos, Ursus americanus*) (Bryan *et al.*, 2015, 2014) offering potential as indicators of pressure due to environmental change and for monitoring of reintroduction success. Our results revealed substantial amounts of progesterone in Iberian lynx hair, measureable both by HPLC-MS/MS and with the progesterone-3CMO-EIA, while testosterone was present in only small amounts.

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Table 1: Comparison of HPLC-MS/MS hormone measurements according to origin and sex

	Median ± inter-quartile range		Mann–Whitney U-test	
Variable	**Captive bred (*n* = 6)**	**Wild caught (*n* = 6)**	**U**	***P*-value**
Cortisol (pg/mg)	8.09 ± 1.37	18.37 ± 8.62	6	0.065
Cortisone (pg/mg)	26.49 ± 7.18	16.50 ± 10.77	28	0.132
DHEA (pg/mg)	29.45 ± 19.14	35.06 ± 27.84	17	0.937
Cortisol/cortisone (ratio)	0.37 ± 0.13	0.98 ± 0.29	4	**0.026**
Cortisol/DHEA (ratio)	0.27 ± 0.01	0.48 ± 0.05	1	**0.004**
Variable	**Male (*n* = 6)**	**Female (*n* = 6)**	**U**	***P*-value**
Cortisol (pg/mg)	7.84 ± 2.95	18.37 ± 8.62	33	**0.015**
Cortisone (pg/mg)	26.16 ± 9.98	20.32 ± 12.68	11	0.309
DHEA (pg/mg)	23.68 ± 10.49	41.19 ± 18.42	27	0.180
Progesterone (pg/mg)	4.00 ± 2.72	13.15 ± 7.62	36	**0.002**
Testosterone (pg/mg)	1.66 ± 0.28	1.82 ± 0.29	26	0.240
Cortisol/cortisone (ratio)	0.28 ± 0.14	0.98 ± 0.29	34	**0.009**
Cortisol/DHEA (ratio)	0.28 ± 0.01	0.48 ± 0.05	30	0.065

HPLC-MS/MS hormone measurements according to origin (six captive born and six wild caught) and sex (six male and six female) in Iberian lynxes held in the captive breeding program. Results are presented as median ± inter-quartile range and Mann–Whitney U-test results for values of cortisol, cortisone, DHEA, progesterone, testosterone, cortisol-cortisone ratio and cortisol-DHEA ratio.

Characterization of the progesterone-3CMO-EIA immunoresponse using HPLC showed immunoreactivity mostly to progesterone and 5α-DHP. The measurement by EIA resulted in a 3-fold overestimation of progesterone in hair when compared to the HPLC-MS/MS results, demonstrating the necessity for validation before assay application. The overestimation for progesterone in hair based on the progesterone-3CMO-EIA is not surprising and easily explained. As mentioned earlier, HPLC-MS/MS offers the advantage of high sensitivity combined with high specificity, allowing the unequivocal identification and quantification of a single compound. The specificity of EIA, however, depends strongly on the cross-reactivity of the antibody. In this case, a high cross-reactivity was reported for 5α-DHP (76.8%) and other progestins. 5α-DHP is a biologically active 5α-reduced metabolite of plasma progesterone and precursor for other progestins. HPLC analysis (Fig. 4) showed a three times higher immunoreactivity for 5α-DHP (46%) compared to progesterone for the progesterone-3CMO-EIA, hence explaining the consistently higher levels measured with EIA compared to HPLC-MS/MS. Nevertheless, the progesterone-3CMO-EIA confirmed to be suitable for reliable analysis of progestins in lynx hair. Progestin levels measured by EIA showed significant differences between groups, with adult females presenting higher levels than other age-sex classes (Fig. 2).

A large difference in circulating progesterone levels between non-breeding females, juveniles and males is not commonly expected for feline species, which usually express an induced ovulation. Active *corpora lutea* are usually only found after mating with elevated progesterone tied to formation of *corpora lutea* and persisting throughout pregnancy (Verhage *et al*., 1976; Swanson *et al*., 1995). Accordingly, hair progesterone is increased during pregnancy in domestic cats in comparison to spayed females (Terwissen *et al*., 2014). In contrast to brown bears (Cattet *et al*., 2017), hair of adult non-pregnant cats contains progesterone levels comparable to pregnancy either indicating a former (recent) luteal activity or a prolonged storage of incorporated progesterone within the hair matrices. The possibility of prolonged stability of progesterone in hair is further supported by the high correlation we found between measurements in different hair segments (Supplementary Table S6). The difference between adult females, irrespective of their actual reproductive status, in comparison to males or juveniles was however expected for females of the lynx genus due to the above-mentioned presence of persistent corpora lutea (Jewgenow *et al*., 2014; Painer *et al*., 2014). In the lynx, elevated serum progesterone levels are found throughout the year, including inactive reproductive periods (Göritz *et al*., 2009). Previous measurements performed on hair from Canada lynx (*Lynx canadensis*) pelts also identified higher progesterone levels in adult females when compared to males, although with some overlap between classes (Terwissen *et al*., 2014). Importantly, the ranges of progesterone values in Iberian lynx females, with all samples collected during the anestrus period (November–December) did not overlap with those of males in any age group in this study.

Like many solitary felids, the Iberian lynx's spatial organization functions as a land tenure system with little or

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no intra-sexual overlap in core areas of each home range (Ferreras *et al*., 1997). Therefore, with further refinement, our data suggest there is potential for identification of territorial adult Iberian lynx females in the field based on progesterone measurement in hair samples. Progesterone values in blood samples of Iberian lynx were 4.3 ng/ml ($n = 19$) and 17.0 ng/ml ($n = 14$) in non-pregnant and pregnant females, respectively (Göritz *et al*., 2009), indicating a 3-fold increase during pregnancy. As a result of opportunistic sampling, our study is limited by the absence of samples from the active reproductive period, but future studies with samples of captive lynxes covering a broad array of the reproductive cycle may characterize hair progesterone variation throughout the year. This study clearly indicates that hair progesterone is a promising indicator of sex and maturity in (female) lynxes. Additionally, understanding how progesterone varies in hair, especially during estrus, pregnancy and lactation or anestrus can potentially provide a non-invasive method for (retrospective) pregnancy diagnosis in the wild. This is especially relevant for estimating reproductive success in elusive species like lynxes.

In contrast to progesterone, hair testosterone is not a suitable indicator of sex since it was detected at very low levels in Iberian lynx hair, both by HPLC-MS/MS and EIA. The latter method yielded a 4-fold overestimation, most likely associated with cross-reacting androgens. The mean testosterone values detected by HPLC-MS/MS in adult Iberian lynx hair samples (2.36 ± 2.04 pg/mg, $n = 12$; no difference between sexes) were similar to those reported in the Canada lynx (3.35 ± 1.65 pg/mg, $n = 45$) based on EIA (Terwissen *et al*., 2014). The mean testosterone values determined with the testosterone-3CMO-EIA were between 5 pg/mg (in juvenile females) and 25 pg/mg (and juvenile males). The HPLC immunograms for our testosterone EIA revealed that besides a small percentage of testosterone (<10%), many unknown hair-borne substances contribute to the EIA values, making the results difficult to interpret. Previously, we clearly demonstrated that lack of antibody specificity may skew targeted hormone analysis and lead to erroneous results (Jewgenow *et al*., 2020). Nevertheless, in this study it is hard to evaluate the specificity of the assay's antibody due to low testosterone levels in lynx hair as confirmed by HPLC-MS/MS. A higher level of testosterone in adult male lynx hair was however expected as outcome of this study. Serum testosterone concentration of Iberian lynx males (0.40 ± 0.22 ng/ml, $n = 11$, range 0.14–0.89 ng/ml) exceeds the levels of females and subadults (below limit of detection of EIA) by several orders (Jewgenow unpublished results, but see also Jewgenow *et al*. (2006) for Eurasian lynx). However, this difference is not reflected in the hair testosterone levels of our Iberian lynx samples or Canada lynx pelts (Terwissen *et al*., 2014). Indeed, sex differences in hair testosterone concentrations were undetectable in most studies in carnivorans (Bryan *et al*., 2014; Terwissen *et al*., 2014; Schell *et al*., 2017). The two exceptions were one study in the grey wolf (*Canis lupus*), where males present higher concentrations than females (Bryan *et al*., 2015) and one in brown bears (*Ursus arctos*), where females had higher testosterone levels than males (Cattet *et al*., 2017). One explanation might be that testosterone is not incorporated into the hair matrix at the same rate as other steroid hormones (or at all) and does not reflect the serum concentration at any time point of the male reproductive cycle. Another possibility is that it is incorporated as another steroid, as described by Kapoor *et al*. (2018) for cortisol and cortisone. Testosterone can be metabolized to estradiol by aromatase and to 5α-dihydrotestosterone (5α-DHT) by 5α-reductase enzymes. 5α-reductase expression has been demonstrated in the skin of several mammal species, where it regulates 5α-DHT effects on hair growth (Yamana *et al*., 2010; Azzouni *et al*., 2012; Robitaille and Langlois, 2020). The fact that the hair follicle is a target organ for testosterone and 5α-DHT could explain the need of a local regulation mechanism to protect follicle function from the potential influence of reproductive fluctuations of serum testosterone. Studies with radiolabeled testosterone could be useful in clarifying this hypothesis.

Regarding glucocorticoids, our HPLC-MS/MS results show overall higher concentrations of cortisone (24.82 ± 9.58 pg/mg) compared to cortisol (13.35 ± 7.80 pg/mg). Growing evidence supports taking cortisol-to-cortisone interconversion into account when studying hGC incorporation. Raúl *et al*. (2004) noted a higher concentration of cortisone in relation to cortisol in hair when compared to blood, and more recently, Kapoor *et al*. (2018) demonstrated that radiolabeled cortisol injected intravenously was incorporated into hair as cortisol, cortisone and unknown glucocorticoid metabolites. Two 11β-hydroxysteroid dehydrogenase (11β-HSD) isoenzymes are known to inter-covert cortisol (active glucocorticoid) and cortisone (inactive glucocorticoid) in several species (Tomlinson and Stewart, 2001). Isoform 11β-HSD-1 converts cortisone to cortisol and is expressed, among other tissues, in the human epidermis, while isoform 11β-HSD-2 converts cortisol to cortisone and is expressed in human sweat glands (Hirasawa *et al*., 1997; Tomlinson and Stewart, 2001). These isoenzymes regulate cortisol availability, enabling the necessary amount of cortisol in circulation and specific organs to ensure homeostasis, while protecting mineralocorticoid receptors from binding with excessive cortisol (Tomlinson and Stewart, 2001). Local cortisol-to-cortisone conversion by 11β-HSD-2 in eccrine sweat glands (Raul *et al*., 2004) and different rates of incorporation of cortisone due to its lower polarity (Raul *et al*., 2004; Kapoor *et al*., 2018) have been suggested to explain the high amount of cortisone present in hair. The absence of functional eccrine sweat glands in hairy skin of mammals (Montagna, 1967) makes the first possibility unlikely in the present case. Additionally, progesterone, a more polar hormone than cortisol and cortisone, showed higher correlation between values of proximal and distal hair segments (Supplementary Table S7), thus suggesting an easier diffusion along the hair shaft. While most studies of

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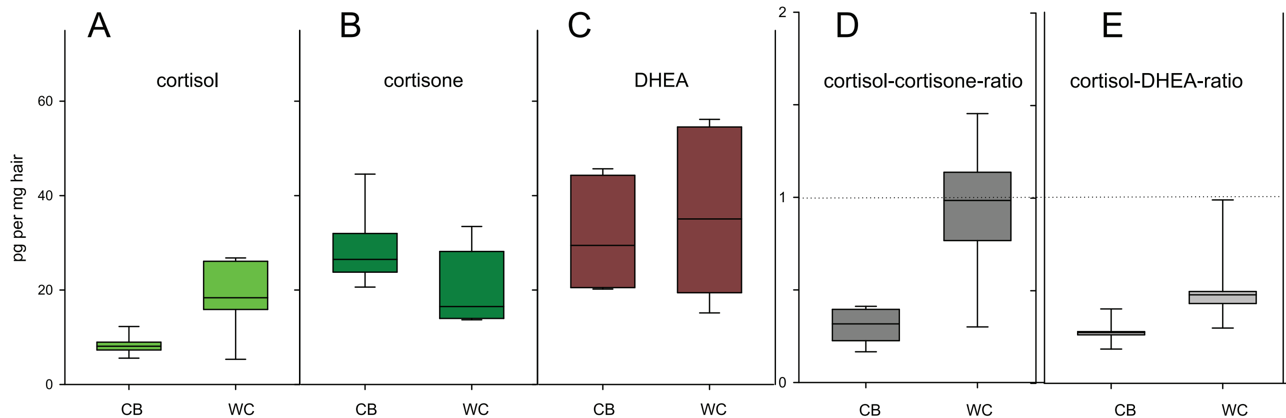

Figure 6: hGCs and their ratios in captive-bred and wild-caught Iberian lynx. Measurements of cortisol, cortisone, DHEA, as well as cortisol-cortisone and cortisol-DHEA ratios calculated from HPLC-MS/MS results, in hair samples of captive-bred ($n = 6$) and wild-caught ($n = 6$) Iberian lynxes

hair steroids in mammals quantified only cortisol (Koren *et al.*, 2019), the higher amounts of cortisone and DHEA that we detected in Iberian lynx hair by HPLC-MS/MS, together with the results of radiolabel validation studies (Keckeis *et al.*, 2012; Kapoor *et al.*, 2018) make it unclear whether the use of an EIA targeting only cortisol is sufficiently informative as a proxy of chronic activation of the stress response in this species. In fact, when investigating the relation of management and behavioral variables on cortisol alone measured by EIA in 19 captive Iberian lynx females, we found no effect (Supplementary Table S5).

Adult males presented significantly higher glucocorticoid levels measured by cortisol-3CMO-EIA compared to all juveniles and higher cortisone EIA values compared to adult females and juveniles. Glucocorticoid levels can fluctuate in mammal species depending on age, sex and season. Based on our previous work with the Egyptian mongoose (Azevedo *et al.*, 2019), we expected hGC measured with cortisol-3CMO-EIA to be higher in young juveniles and in males, when compared to other age-sex groups. The conflicting results we observe for young Iberian lynx are probably due to the fact that all juveniles in our sample are older than 6 months of age and beyond the post-weaning phase where increased levels of hGC were observed in the mongoose. However, the cortisol-3CMO-EIA measurements also conflict with the HPLC-MS/MS measurements, where we found higher cortisone in males compared to females, but the opposite for cortisol. The cross-reaction of the cortisol-3CMO-EIA with cortisone and the proven presence of cortisone in lynx hair (see HPLC immunogram in Fig. 4) partially explain this conflict by contributing to an overestimation of EIA cortisol measurements in females and an underestimation in males. A comparison of the HPLC-MS/MS and EIA results for 12 Iberian lynx hair samples shows a 2-fold overestimation with the cortisol-3CMO- EIA, whose values were similar and significantly correlated to the sum of cortisone and cortisol obtained by HPLC-MS/MS. This is yet another example of where choosing the wrong assay can lead to erroneous results (Jewgenow *et al.*, 2020).

DHEA was the most abundant steroid in Iberian lynx hair. This androgen with anti-glucocorticoid effects is produced in the adrenal glands and the brain (Maninger *et al.*, 2009) and has been proposed as an indicator of allostatic load in humans (Mcewen, 2004). The mechanisms by which DHEA exerts its anti-glucocorticoid effects are not fully known, but it has been shown to decrease the expression of 11β-HSD-1 and increase the expression and activity of 11β-HSD-2 in mice, both *in vivo* and in cell cultures (Maninger *et al.*, 2009). In human skin samples, its metabolites 7α- and 7β-hydroxy-DHEA competitively inhibit cortisone binding to 11β-HSD-1 and consequently its conversion to cortisol (Hennebert *et al.*, 2007). In a similar approach as the one used for cortisone, several studies in humans showed serum and salivary cortisol-DHEA ratios to be more accurate predictors of depression than DHEA or cortisol levels alone (Maninger *et al.*, 2009).

We calculated cortisol-cortisone and cortisol-DHEA ratios in Iberian lynx hair, and assessed their variation between animals of different origin and sex, in comparison to the values of the single hormones (Table 1, Fig. 6). While trends are noticeable using the single hormones, with apparently higher cortisol and lower cortisone concentrations in wild-caught Iberian lynxes of the captive breeding program compared to their captive-bred counterparts, these differences were not statistically significant. However, when combining cortisol and cortisone values into a cortisol-cortisone ratio, the differences between captive-bred and wild-caught animals become more evident (Fig. 6) and statistically significant (Table 1). More surprisingly, despite no apparent difference in DHEA levels between both groups, the cortisol-DHEA ratio was different between wild-caught and captive-bred Iberian lynxes, with the highest statistical significance of all variables tested. These findings are consistent with the hypothesis of a balanced mechanism using cortisone and cortisol interconver-

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sion through 11β-HSD enzymes regulated by DHEA. With regards to the above conclusions in relation to the animals' origin, it is important to explain the opportunistic nature of our sample collection with five out of six wild-caught animals being females, one male and vice-versa in the captive-bred group. The wild-caught status is expected to be associated with higher stress levels in captivity, but the latter hypothesis is difficult to prove unequivocally in this study because of the potential sex bias. This again illustrates the difficulty in controlling for confounding factors when working with critically endangered species. Nevertheless, since the activity of 11β-HSD-1 and its regulation by DHEA metabolites has been demonstrated in human skin samples (Hennebert *et al.*, 2007) and radiolabeled cortisol has been shown to be incorporated in hair as cortisone and other metabolites (Kapoor *et al.*, 2018), it is difficult to dismiss the possibility that glucocorticoid levels found in Iberian lynx hair samples could be the end result of a similar system, possibly as part of a local HPA-axis analogue (Slominski *et al.*, 2007). Unfortunately, most validation studies using repeated ACTH challenge (e.g. del Rosario *et al.*, 2011; Terwissen *et al.*, 2013) and known stressors (e.g. Davenport *et al.*, 2006) have been performed using only cortisol assays. Further studies comparing the simultaneous variations in hair cortisol, cortisone and DHEA in response to repeated ACTH challenge and known stressors will be necessary to clarify this.

Finally, in order to explore the practical applicability of this concept, we compared the cortisol-3CMO-EIA and cortisone-EIA measurements for an Iberian lynx after a 24-day escape following a wildfire that affected the breeding center. In the 3 weeks following the escape-recapture event, there was an inversion of the ratio of the cortisol-3CMO-EIA to cortisone-EIA measurements, indicating a relative rise in cortisol concentration compared to cortisone (Fig. 3). Approximately 1 year later, the trend had inverted again, with higher cortisone-EIA measurements. Despite the limitation of the cortisol-3CMO-EIA, lacking specificity by partial cross reactivity with cortisone, the results do support the need for further research into the potential value of more than one steroid as an indicator of stress in this species.

Hormone measurement in hair using EIA can provide valuable tools for wildlife management and conservation. Limitations such as the lack of specificity due to antibody cross-reactivity need to be accounted for by prior cross-validation by HPLC and HPLC-MS/MS, as we demonstrate for progesterone and cortisol. Nevertheless, a more comprehensive approach that accounts for biologically relevant local metabolic pathways might be necessary to achieve the full potential of hair steroid measurement, as is becoming increasingly evident in the case of hGCs.

Supplementary material

Supplementary material is available at *Conservation Physiology* online.

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5. Article 4 – variation of hair glucocorticoids in wild Egyptian mongoose

Azevedo A, Bailey L, Bandeira V, Fonseca C, Wauters J & Jewgenow K (2021). Decreasing glucocorticoid levels towards the expansion front suggest ongoing expansion in a terrestrial mammal. *Conservation Physiology*, 9(1), coab050. https://doi.org/10.1093/conphys/coab050

The aim of this research was to investigate how spatial and environmental factors influence long-term adrenocortical activity in a wild population of Egyptian mongoose (*Herpestes ichneumon*) that has been expanding its range over the last thirty years. For that purpose, we measured glucocorticoids in Egyptian mongoose hair samples from the species' entire range within Portugal, using our previously validated EIA. Next, we applied an information theoretic approach to determine which spatial (historic vs. expansion region, distance to Tagus river within the expansion population) and environmental [area of favourable habitat, European rabbit (*Oryctolagus cuniculus*) harvest data, Egyptian mongoose harvest data, human population density] factors influenced hair glucocorticoids, after accounting for normal variation with age, sex, season, body size, body condition and sample storage time.

Author contributions:

AA: conception and design, laboratory analysis and data acquisition, analysis and interpretation, writing, review and editing; LB: analysis and interpretation, writing, review and editing; VB: data acquisition, review and editing; CF review and editing; JW: analysis and interpretation, review and editing; KJ: design, analysis and interpretation, review and editing, supervision.

Results of the study:

1. We observed a decrease in hair glucocorticoids towards the expansion front *i.e.,* as the distance from the Tagus River, which was thought to limit the species historic range, increased.

2. Hair glucocorticoids were estimated to decrease 0.031 pg/mg (95% CI: -0.057, -0.004) for each additional kilometre of distance to the Tagus River, which corresponds to an estimated 1.0 pg/mg decrease for every 32 km.
3. There was strong evidence for an effect of body size and body condition on hair glucocorticoid levels, which could be at least partially responsible for glucocorticoid variation with age that we had previously observed.
4. Normal patterns of variation with age, sex, body condition and body size, had strong effect compared to spatial and environmental variables, as did storage time. These results illustrate the importance of accounting for the effect of these variables when attempting to measure stress.
5. There was little evidence of an effect of human population density or area of favourable habitat on mongoose hair glucocorticoids, possibly due to the behavioural plasticity that characterizes the mongoose genus.
6. Overall, the decrease of hair glucocorticoids toward the expansion front and the lack of an effect of human density and favourable habitat suggest the Egyptian mongoose expansion is unlikely to be limited by mechanisms related to- or mediated by the physiological stress response.

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SEB SOCIETY FOR EXPERIMENTAL BIOLOGY

Research article

Decreasing glucocorticoid levels towards the expansion front suggest ongoing expansion in a terrestrial mammal

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Understanding the causes of range expansions in abundant species can help predict future species distributions. During range expansions, animals are exposed to novel environments and are required to cope with new and unpredictable stressors. Glucocorticoids (GCs) are mediators of the hormonal and behavioural mechanisms allowing animals to cope with unpredictable changes in the environment and are therefore expected to differ between populations at expansion edge and the historic range. However, to date, very few studies have evaluated the relationship between GCs and range expansion. The Egyptian mongoose has been rapidly expanding its range in Portugal over the past 30 years. In this study, we applied an information theoretic approach to determine the most important spatial and environmental predictors of hair GCs (hGCs) in the population, after controlling for normal patterns of hGC variation in the species. We observed a decrease in hGC as distance from the historic range increased (i.e. closer to the expansion front). This distance term was present in all of the top models and had a 95% confidence interval (95% CI) that did not overlap with zero, strongly supporting its influence on hGC. We estimated a 0.031 pg/mg (95% CI: −0.057, −0.004) decrease in hGCs for each kilometre distance to the Tagus River, which was once the limit of the species' distribution. Our results indicate that the species' expansion is unlikely to be limited by mechanisms related to or mediated by the physiological stress response. The decrease in hGC levels towards the expansion edge coupled with limited evidence of a negative effect of human population density suggests that the species' northward expansion in Portugal could continue.

Key words: Egyptian mongoose, hair glucocorticoids, *Herpestes ichneumon*, range expansion, stress

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Introduction

The abundance, richness and spatial distribution of naturally occurring populations of wild species are changing at an unprecedented rate as a consequence of anthropogenic environmental change (Pimm *et al*., 2014; Newbold *et al*., 2015). Ecologists and evolutionary biologists have sought to understand the factors that shape species' spatial distributions for decades (Holt, 2003; Liebl and Martin, 2012). In the Anthropocene, a small number of species have expanded their ranges, contradicting the general trend of decline driven by anthropogenic environmental change (Liebl and Martin, 2012). While the study of these exceptions can offer important information to predict how populations respond to environmental change, opportunities for such studies are rare (Hui *et al*., 2012; Liebl and Martin, 2012, 2013).

Range expansions take place through the dispersal of individuals from the core population into novel environments. This is influenced by the set of environmental factors that enable the species to persist (niche), their spatial distribution (habitat) and the ability of the species to move (disperse) and adapt to new areas (Holt, 2003). Range expansions can take place when species occupy niche opportunities that arise due to changes in biotic or abiotic factors, such as changes in climate, land use or extirpation of competing species, or alternatively, due to the emigration of individuals from core populations into habitats where their niche requirements are not met, creating sink populations (Holt, 2003). The neuroendocrine stress response allows organisms to cope with unpredictable stressors in the environment (Wingfield *et al*., 1998). Accordingly, the colonization of new and unfamiliar environments during range expansions is expected to be facilitated by increased stress reactivity (Liebl and Martin, 2012; Martin *et al*., 2017). However, if the new and unknown environment leads to very frequent or chronic activation of the stress response, impacts of elevated glucocorticoids (GCs) on survival, reproduction and fitness may occur (Sapolsky *et al*., 2000; Bonier *et al*., 2009a).

In vertebrates, GCs are released by activation of the hypothalamic–pituitary–adrenal axis (HPA-axis) in response to challenging environmental stimuli (Wingfield *et al*., 1998; Sapolsky *et al*., 2000). This neuroendocrine response allows animals to respond to environmental cues (Wingfield and Mukai, 2009) and adjust their physiology and behaviour to cope with and recover from unpredictable environmental change (Wingfield *et al*., 1998; Sapolsky *et al*., 2000; Zimmer *et al*., 2020). Due to the pervasive effects of GCs, chronic elevations are thought to result in deleterious effects on survival, reproduction (Sapolsky *et al*., 2000) and fitness (Breuner *et al*., 2008; Vitousek *et al*., 2018). However, relations between baseline and stress-induced blood and faecal GC levels and fitness have been inconsistent (Bonier *et al*., 2009a, 2009b; Dantzer *et al*., 2014) and context dependent (Creel *et al*., 2013; Vitousek *et al*., 2018).

Differences in HPA-axis physiology could influence the ability of animals to colonize new environments during range expansion, but studies evaluating this link are scarce and focus on birds, reptiles and amphibians (e.g. Atwell *et al*., 2012; Liebl and Martin, 2012; Brown *et al*., 2015; Martin *et al*., 2018). For example, in expanding populations of house sparrows (*Passer domesticus*), individuals at the range edge have been shown to exhibit increased stress-induced GC levels and differences in the expression of the receptors involved in GC pathways, which is thought to facilitate their ability to colonize novel environments (Liebl and Martin 2012, 2013; Martin *et al*., 2017). In dark-eyed juncos (*Junco hyemalis*) and cane toads (*Rhinella marina*), a difference in GC reactivity was also observed between individuals of the newly established and historic populations, but with the colonists or edge populations showing decreased stress-induced GC levels (Atwell *et al*., 2012; Brown *et al*., 2015).

Baseline levels of GCs during range expansions have also shown inconsistent trends (Atwell *et al*., 2012; Liebl and Martin, 2012; Martin *et al*., 2017). A broad scale study including approximately one hundred species of birds and reptiles found little evidence of a relation between stress-induced or baseline GCs and edge/non-edge location within the population (Martin *et al*., 2018). The inconsistency of these findings could be due to methodological factors, such as the lability of point samples of blood GC measurements (Bonier *et al*., 2009a). Baseline GCs based on plasma samples have shown low intra-individual repeatability (Vitousek *et al*., 2018, 2019b) compared to GC measurements from matrices reflecting long-term GC levels (Taff *et al*., 2018) and are greatly influenced by environmental conditions. Alternatively, these inconsistencies could be explained by the context dependence of stress-induced and basline levels of GC (Vitousek *et al*., 2018) or the fact that they are simply separate traits shaped by different selective pressures (Vitousek *et al*., 2019a).

Hair GC (hGC) measurements are thought to reflect both baseline and stress-induced GCs incorporated into hair over prolonged periods (D'Anna-Hernandez *et al*., 2011; Kapoor *et al*., 2018; Short *et al*., 2016) and suffer little influence from short-term variations such as those caused by capture or handling. This may allow the identification of long-term trends in overall GC exposure that would be difficult to detect using matrices reflecting short-term variations. For example, a wild population of red deer (*Cervus elaphus*) exhibited an increase in hGC levels in response to hunting activity that was not detectable in faeces, while baseline plasma GC levels actually tended to decrease (Vilela *et al*., 2020). Hence, information on long-term GC exposure obtained from hGC analysis could help understand range expansions and predict whether expanding populations are likely to become established and pose an invasion risk (Martin *et al*., 2017) or result in sink populations.

The Egyptian mongoose (*Herpestes ichneumon*) is a medium sized carnivore that is widely distributed across

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Africa and the Middle East. In Europe, it is only present in the Iberian Peninsula, most likely due to colonization in the Late Pleistocene (Gaubert *et al*., 2011). In the past three decades, the species has rapidly increased its range to the north of the Tagus River (Fig. 1), which was once considered a natural barrier to its expansion (Barros *et al*., 2016b). In the expansion area, the species experiences very different environmental conditions, such as higher human density and primary productivity, and lower availability of favourable habitat (Bandeira *et al*., 2016). Based on presence–absence data, changes in land use such as rural abandonment have been identified as drivers of the expansion of the Egyptian mongoose (Barros *et al*., 2015). Morphological differences have been identified between the populations inhabiting the historic and expansion areas, with lower size (Bandeira *et al*., 2016) and body condition (Bandeira *et al*., 2019) and higher testicular mass (Bandeira *et al*., 2021) in the expansion area. However, no information exists on GC levels across the species' range.

In order to be informative, GC measures require species-specific validation (Touma and Palme, 2005; Azevedo *et al*., 2020; Jewgenow *et al*., 2020) as well as consideration of how 'normal' patterns of variation (such as age, sex and season) and interacting environmental factors influence the physiological response (Dantzer *et al*., 2014). Both cortisol and cortisone are stress hormones that have been identified in Egyptian mongoose hair (Jewgenow *et al*., 2020). In previous work, we cross-validated an enzyme immunoassay (EIA) targeting cortisol and cortisone in guard hairs of this species with liquid chromatography coupled with mass spectrometry (LC–MS/MS) and high-pressure liquid chromatography (HPLC) (Jewgenow *et al*., 2020) and characterized normative variations with age, sex and season within the free-ranging population inhabiting Portugal (Azevedo *et al*., 2019).

In the current study, we measured hGC in Egyptian mongoose over the species' entire range within Portugal. We applied an information theoretic approach (Burnham and Anderson, 1998) to determine which spatial (historic vs. expansion region, distance to Tagus river within the expansion population) and environmental factors [area of favourable habitat, European rabbit (*Oryctolagus cuniculus*) harvest data, Egyptian mongoose harvest data, human population density] from our data set influence hGC levels in the population, while controlling for known effects of age, sex, season and sample storage time. To our knowledge, this is the first study assessing the relation of GC with a mammalian range expansion and the only study using integumentary long-term GC measurements. If higher long-term GC levels facilitate expansion just as stress-induced blood GC levels seem to (Liebl and Martin, 2012, 2013; Martin *et al*., 2017), we expect to see higher hGC levels in the expansion area, with tendency to increase as the distance to the Tagus river increases. Alternatively, a decrease in hGC levels towards the expansion front would be more likely in a scenario where animals in the expansion area were presented with less frequent or severe stressors or where lower baseline hGCs favoured colonization.

Methods

Sample collection

We obtained data and hair samples from 294 carcasses of wild Egyptian mongoose collected throughout the year, between January 2008 and December 2014 from hunting activities throughout mainland Portugal (Bandeira *et al*., 2016). After the exclusion of specimens for which data were missing, 236 samples remained and were used in this study. Carcasses were stored frozen at −20°C and then thawed at the time of sample collection. Hair was clipped with scissors as close to the skin as possible from a standard location (between the 'scapulae') in order to account for variation in hGC with anatomical region (Azevedo *et al*., 2019). Hair samples were stored in paper envelopes in a dry and dark location until the date of GC extraction.

hGC measurement

hGCs were quantified by an EIA for which validation was previously performed (Azevedo *et al*., 2019). Briefly, 20 mg of guard hairs were separated from undercoat, washed twice with 90% methanol for 5–10 seconds and dried at 70°C. Next, 10 mg of hair were weighed and ground to a fine powder in a Precellys24 tissue homogenizer (Bertin Technologies, France). Finally, GCs were extracted from the powdered hair with 90% methanol and centrifuged, and the supernatant was collected and frozen until the day of GC measurement. hGCs were measured with an EIA using a polyclonal antibody (rabbit) against cortisol-3-CMO-BSA and cortisol-3-CMO-peroxidase as label. The assay was validated by demonstrating parallelism of serially diluted hair extracts to the cortisol standard curve, and the inter-assay coefficients of variation were 10.78% for extracts containing low and 15.95% for extracts containing high concentrations of cortisol. The intra-assay coefficients of variation were 6.72% for extracts containing low and 5.37% for extracts containing high concentrations of cortisol and the sensitivity of the assay was 0.40 pg/well. In order to determine if the EIA was targeting the intended steroids, it was validated by HPLC analysis that demonstrated that the cortisol-3CMO antibody was binding to cortisol and small amounts of cortisone, and finally by demonstrating a strong correlation between cortisol-3CMO-EIA measurements and HPLC-MS/MS measurements of cortisol and cortisone from the same extracts. The validation of the EIA is reported in detail in Azevedo *et al.* (2019).

Spatial variation

In order to assess how expansion is related to hGC levels, Egyptian mongoose specimens were assigned to the 'historic' region if they were captured south of the Tagus River, or to the 'expansion' region if they were captured north of the Tagus

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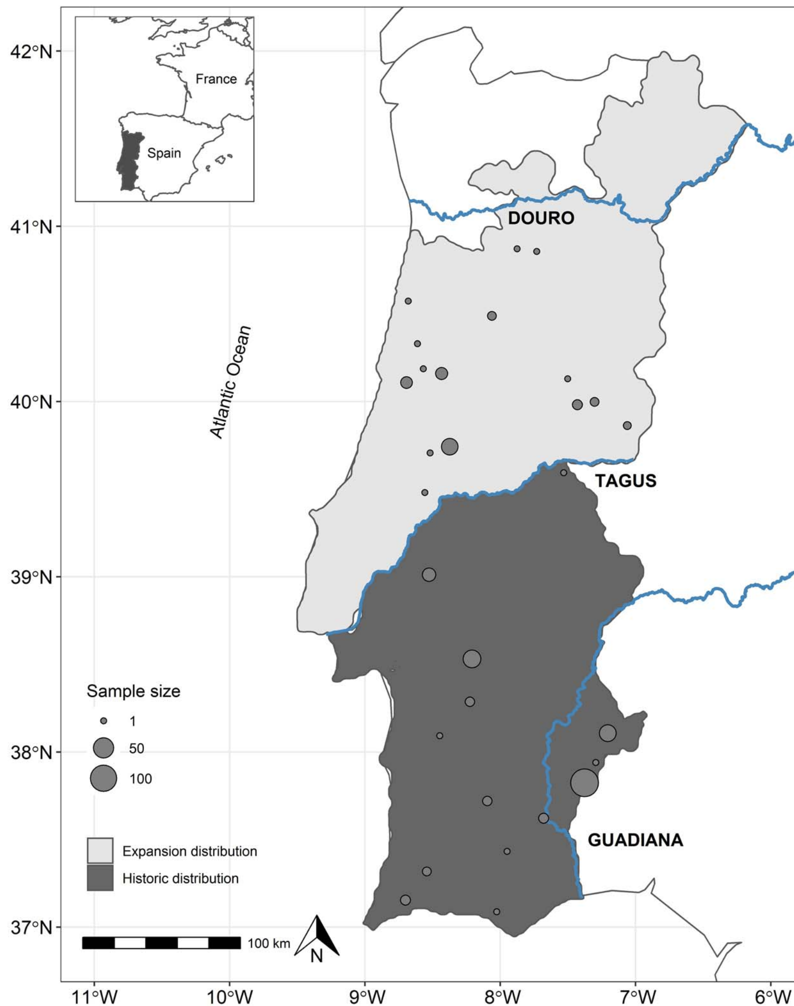

Figure 1: Geographic distribution of the Egyptian mongoose in Portugal. The species was confined to the South of the Tagus River (dark grey area). In the past three decades, it has been rapidly expanding northward (light grey area). Circles represent number of specimens sampled in each location.

River (Fig. 1). For specimens captured in the 'expansion' region, the shortest distance from capture location to the Tagus River in kilometres was calculated, and the resulting variable 'distance to river' was included in model construction to assess whether being closer to the expansion edge (or further from the core population) influenced hGC levels.

Normative patterns of variation

In our previous study age, sex and storage time were shown to influence hGC measurements in this species (Azevedo *et al.*, 2019). hGC levels were higher in males compared to females and in juveniles younger than 5.5 months compared to other age cohorts and decreased with storage time (Azevedo *et al.*, 2019). Therefore, the effect of these variables was accounted for by including them in the model. Each mongoose was classified as an adult (over 1 year of age), sub-adult (between 9 and 12 months), type 2 juvenile (between 5.5 and 9 months) and type 1 juvenile (between 2.5 and 5.5 months of age) based on dental development (Bandeira *et al.*, 2016). Specimens were designated as male or female based on the presence of testicles or ovaries. Storage time was defined as the total number of days between the date of capture of the mongoose and the date of cortisol extraction from hair (1150 to 2266 days). Although not a significant factor in our prior analyses (Azevedo *et al.*, 2019), seasonal variations in GCs have often been demonstrated in vertebrates (Romero, 2002). Season was included in our model to account for the species' seasonal reproductive activity with a peak in spring, which is possibly delayed in the expansion region (Bandeira *et al.*, 2021). Animals were assigned to winter (January to March), spring (April to June), summer (July to September) or autumn (October to December) according to date of capture. We included snout–tail length (STL) values obtained by standard

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mammal measurement methods to account for the potential effect of metabolic rate on baseline GCs, and because smaller animals may have less energy reserves and hence require enhanced GC responsiveness to meet unpredictable energy demands (Haase *et al.*, 2016; Francis *et al.*, 2018; Vitousek *et al.*, 2019a). Finally, we included an index of body condition score (BCS) to account for the amount of energy reserves present in each specimen at the time of capture. We expect body condition to influence GC levels differently from size because of the central role of GCs in the regulation of energy metabolism (Sapolsky *et al.*, 2000). For calculation of the BCS, we used the 'scaled mass index' based on body mass scaled for STL (Peig and Green, 2009, 2010).

Environmental factors

The environmental factors used for model construction were selected based on our predictions of their biological relevance for hGC measurement drawn from results of previous studies with the species (Barros *et al.*, 2015, 2016a; Bandeira *et al.*, 2016, 2018, 2019). All environmental variables were presented as mean values within the 2×2 km grid cell where the specimen was collected. The reported home range size for the Egyptian mongoose in the Iberian Peninsula is 3.10 ± 2.12 km^2 (Palomares, 1994). Hence, the grid cell area of 4 km^2 is likely to offer an approximation of the environmental conditions each specimen experiences in its territory. We used the area occupied by shrub and/or agroforestry habitat in each grid cell to obtain a proxy of the availability of habitat types that have been shown to favour gene flow (Barros *et al.*, 2016a) and expansion (Barros *et al.*, 2015) of the species in Portugal. Our prediction was that habitat types that have been favourable to the species' expansion would be associated with lower hGC levels. Human population density presented as the number of inhabitants per km^2 in each grid cell (data from Eurostat) (European Commission, 2015) was included as a fixed factor because increased levels of GCs are generally observed with increasing human disturbance (Dantzer *et al.*, 2014). The extent of road network represented by the total length of road in metres in each grid cell (IGP, Instituto Geográfico Português, 2015) was included as a candidate factor in the model but was excluded due to collinearity with human population density. We included the number of Egyptian mongoose reported from hunting bags for each grid cell in the year and month (ICNF, unpublished data) of each specimen's capture as a proxy of relative abundance of conspecifics to account for the effect of mongoose density on GCs. Population density can influence circulating GC levels in vertebrates, especially in non-social territorial species like the Egyptian mongoose where the frequency of social interactions at higher densities leads to more frequent activation of the HPA-axis (Creel *et al.*, 2013). Similarly, European rabbit (*O. cuniculus*) yields for each grid cell in the respective year and month (ICNF, unpublished data) were used as a proxy of relative prey availability because food scarcity or unpredictability may influence GC levels directly (Fokidis *et al.*, 2012).

Statistical methods

We analysed the effect of spatial and environmental factors on Egyptian mongoose hGC using linear mixed effects models with a Gaussian error distribution. Input variables were standardized on two standard deviations to account for differences in scale and to enable comparison of effect sizes (Gelman, 2008; Schielzeth, 2010). Variance inflation factors (VIFs) were used to test for multi-collinearity between variables with a cut-off value of 4. Collinearity was detected between road network (VIF = 6.50) and human population density (VIF = 5.16), which were highly correlated ($r_{(234)} = 0.86$, $P < 0.001$). We considered the latter a more robust measure of human presence, as it is likely to include the effect of road network and many other factors. Therefore, road network was excluded from further analyses. The global model included the effect of the fixed factors age, sex, season STL, BCS, storage time, region, distance to river, favourable habitat, human population density, relative prey availability and relative conspecific density on hGC concentration. We also included the interactions between STL and both sex and age to account for differing effects of body size according to age cohort or sex. Year of capture was included as a random factor to account for non-independence and differences in GC levels in different years. Residuals of the fitted model were visually inspected by plotting against fitted values and with a Q-Q plot, to check model assumptions. We identified and removed two outlier hGC values that were more than six standard deviations from the mean and were causing violations in assumptions of homoscedasticity and normality of residuals. We cannot rule out the possibility that these values are the result of severe stressors, since 4-fold increases in hair cortisol have previously been documented (del Rosario *et al.*, 2011; Mastromonaco *et al.*, 2014). However, we did not consider the effect of these potentially severe and rare stressors useful to answer our current research questions. After outlier removal, residuals of the fitted model displayed an approximately normal distribution with no strong pattern of over-dispersion or heteroscedasticity. The candidate model set included 6656 models that were ranked based on AICc (ΔAICc from the best model ≤ 2.0) (Burnham and Anderson, 1998). We determined the relative importance of each factor using the sum of Akaike weights (*sw*) in the entire candidate model set, with 1 being the most important and 0 the least important. Factors that appeared in a higher number of models from the top model set and had higher sum of weights were considered more likely to be contained in the model best approximating the truth. We performed model averaging (Burnham and Anderson, 2002; Lukacs *et al.*, 2010) on the top model set (ΔAICc ≤ 2.0; Grueber *et al.*, 2011) to account for uncertainty in model selection and obtain more robust estimates. Statistical analyses were performed using the R statistical system v 4.0.3 (R Core Team, 2020); model selection for mixed models was conducted using 'lme4' package (Bates *et al.*, 2015) and 'MuMIn' package for model selection (Barton, 2020).

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Table 1: hGC values (mean ± SD in pg/mg) and number of Egyptian mongoose specimens from each region, age cohort and sex included in statistical analyses

	Historic region 19.03 ± 5.68 (n = 173)		Expansion region 18.83 ± 4.65 (n = 61)	
	Female 18.44 ± 4.28 (n = 91)	Male 19.68 ± 6.88 (n = 82)	Female 18.01 ± 4.39 (n = 34)	Male 19.84 ± 4.84 (n = 27)
Adult 17.78 ± 4.74 (n = 141)	18.49 ± 4.26 (n = 56)	19.16 ± 5.47 (n = 46)	17.84 ± 4.75 (n = 24)	20.23 ± 3.96 (n = 15)
Sub-adult 19.42 ± 6.35 (n = 24)	17.1 ± 2.4 (n = 10)	24.97 ± 9.47 (n = 7)	16.38 ± 2.11 (n = 4)	18.24 ± 2.45 (n = 3)
Juvenile 2 16.99 ± 4.83 (n = 39)	16.77 ± 4.12 (n = 11)	16.62 ± 4.48 (n = 20)	18.5 ± 2.94 (n = 4)	17.96 ± 9.85 (n = 4)
Juvenile 1 22.11 ± 7.02 (n = 30)	20.53 ± 4.9 (n = 14)	25.07 ± 10.65 (n = 9)	22.32 ± 5.35 (n = 2)	21.15 ± 3.7 (n = 5)

Results

Data for a total of 234 specimens were included in the analysis (Table 1). Among these, 141 belonged to the adult cohort, 24 to the sub-adult, 39 to juvenile 2 and 30 to juvenile 1, with a balanced distribution of females (125) and males (109). A total of 173 specimens were captured in the historic region and 61 were captured in the expansion area. hGC levels in Egyptian mongoose hair had a mean of 18.98 ± 5.42 pg/mg and varied between 8.07 and 43.36 pg/mg.

Model selection resulted in 6656 candidate models (Table S1), with a set of 16 models with ΔAICc ≤ 2.0 (Table 2). The distance to the Tagus River for specimens collected in the expansion area appeared in all of the top models and had a relative importance of 0.69 (Table 3). In terms of environmental variables, relative mongoose and rabbit availability were present in most of the top models and had moderate relative importance based on sum of Akaike weights. There was little evidence in support of an effect of human population density, relative area of favourable habitat and region on hGC levels. Age, sex, body condition, body size and sample storage time appeared in all or the majority of the top models and had sums of Akaike weights higher than 0.60, providing evidence for their influence on hCG levels.

Based on the estimates obtained by model averaging using untransformed data (Table 4), hGCs were estimated to decrease 0.031 pg/mg (95% CI: −0.057, −0.004) for each 1 kilometre increase in distance from the capture location to the Tagus River, in the expansion area (Fig. 2). In terms of environmental factors, hGCs were predicted to decrease 0.258 pg/mg (95% CI: −0.513, −0.002) for each additional mongoose harvested in the 2 × 2 km grid cell during that month and to increase 0.011 pg/mg (95% CI: 0.000, 0.023) with each harvested rabbit, after accounting for storage time and normative patterns of variation in the species.

Model averaged effect sizes within the top models (ΔAICc ≤ 2.0; Table 4) indicated that STL had the strongest effect on hGC concentration (−5.20), followed by age (−4.23, juvenile type 2), sample storage time (3.65), sex (2.08, male), distance to the Tagus River within the expansion area (−1.76) and BCS (−1.76). The effects of the number of harvested mongoose (−1.35) and rabbits (1.19), as well as season (−1.43, summer) and human population density (1.22), were weak when compared to the other factors. Interaction of age and sex with STL, region and favourable habitat were not present in the top model set.

Discussion

In this study, we aimed to determine which spatial and environmental factors influence long-term adrenocortical activity in the Egyptian mongoose population in Portugal, in order to better understand its idiosyncratic expansion in the context of anthropogenic change.

We found support for a relation of hGC levels with range expansion in the Egyptian mongoose. The distance between capture location and the Tagus River, in animals from the expansion area, appeared in all models with ΔAICc ≤ 2.0 and had a sum of Akaike weights of 0.69, strongly supporting its inclusion in the model best approximating the truth. Based on the averaged estimates of the top 16 models using standardized input variables, the effect size of the distance to the Tagus (−1.76) was of a magnitude comparable to the effect of sex (2.08) or body condition (−1.76) with a 95% confidence interval that did not include zero (−3.26, −0.26). hGC levels are estimated to decrease 0.031 pg/mg per kilometre as the

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Table 2: Model selection table; top ranked models with ΔAICc ≤ 2.0 are shown here (for full model selection table see Table S1); R^2 and adjusted R^2 (Adj. R^2) values for each model are included (Nakagawa and Schielzeth, 2013); *w* indicates model weights

Age	Region	Sex	Season	Mongoose	Rabbit	BCS	Habitat	Population	STL	Storage	Age:STL	D. river	Sex:STL	R^2	Adj.R^2	df	AICc	ΔAICc	*w*
+	–	2.10	+	−1.14	1.27	−1.86	–	1.09	−5.34	−3.78	–	+	–	0.33	0.33	17.00	1397.29	0.00	0.017
+	–	2.11	+	−1.44	1.28	−1.91	–	–	−5.62	−3.64	–	+	–	0.32	0.32	16.00	1397.34	0.05	0.016
+	–	2.09	+	−1.20	1.23	−1.77	–	1.09	−5.05	−3.84	–	+	−1.74	0.34	0.34	18.00	1397.50	0.22	0.015
+	–	2.10	+	−1.50	1.24	−1.81	–	–	−5.33	−3.70	–	+	−1.74	0.33	0.33	17.00	1397.56	0.27	0.015
+	–	2.01	+	–	1.18	−1.73	–	1.43	−5.17	−3.94	–	+	–	0.32	0.32	16.00	1397.84	0.55	0.013
+	–	2.00	+	–	1.14	−1.64	–	1.45	−4.89	−4.00	–	+	−1.61	0.33	0.33	17.00	1398.36	1.07	0.010
+	–	2.17	–	−1.47	1.08	−1.58	–	1.16	−5.42	–	–	+	–	0.30	0.30	13.00	1398.82	1.53	0.008
+	–	2.00	+	−1.10	–	−1.77	–	1.11	−4.67	−3.93	–	+	−1.84	0.33	0.33	17.00	1399.05	1.76	0.007
+	–	2.01	+	−1.03	–	−1.87	–	1.11	−4.96	−3.87	–	+	–	0.32	0.32	16.00	1399.07	1.79	0.007
+	–	1.94	+	–	–	−1.75	–	1.42	−4.83	−4.01	–	+	–	0.31	0.31	15.00	1399.09	1.80	0.007
+	–	2.16	–	−1.52	1.06	−1.49	–	1.15	−5.15	–	–	+	−1.67	0.30	0.31	14.00	1399.14	1.85	0.007
+	–	2.01	+	−1.41	–	−1.82	–	–	−4.95	−3.79	–	+	−1.84	0.32	0.32	16.00	1399.20	1.91	0.006
+	–	2.02	+	−1.34	–	−1.92	–	–	−5.25	−3.73	–	+	–	0.31	0.31	15.00	1399.21	1.93	0.006
+	–	2.20	–	−1.55	1.02	−1.57	–	1.23	−5.26	−1.96	–	+	–	0.30	0.30	14.00	1399.25	1.96	0.006
+	–	2.18	–	−1.61	1.00	−1.47	–	1.23	−4.95	−2.09	–	+	−1.81	0.31	0.31	15.00	1399.25	1.96	0.006
+	−0.78	2.07	+	−1.43	1.26	−1.88	–	–	−5.65	−3.67	–	+	–	0.32	0.33	17.00	1399.29	2.00	0.006

Legend: BCS, body condition score; STL, snout–tail length; Age:STL, interaction between age and snout–tail length; D. river, distance to the Tagus River for animals captured in the expansion region; sex:STL, interaction between sex and snout–tail length; df, degrees of freedom; AICc, Akaike information criteria; ΔAICc, difference in AICc to the model with lowest AICc; w, Akaike weight. '+' indicates a categorical variable that was included in the model, while '−' indicates a variable that was not present.

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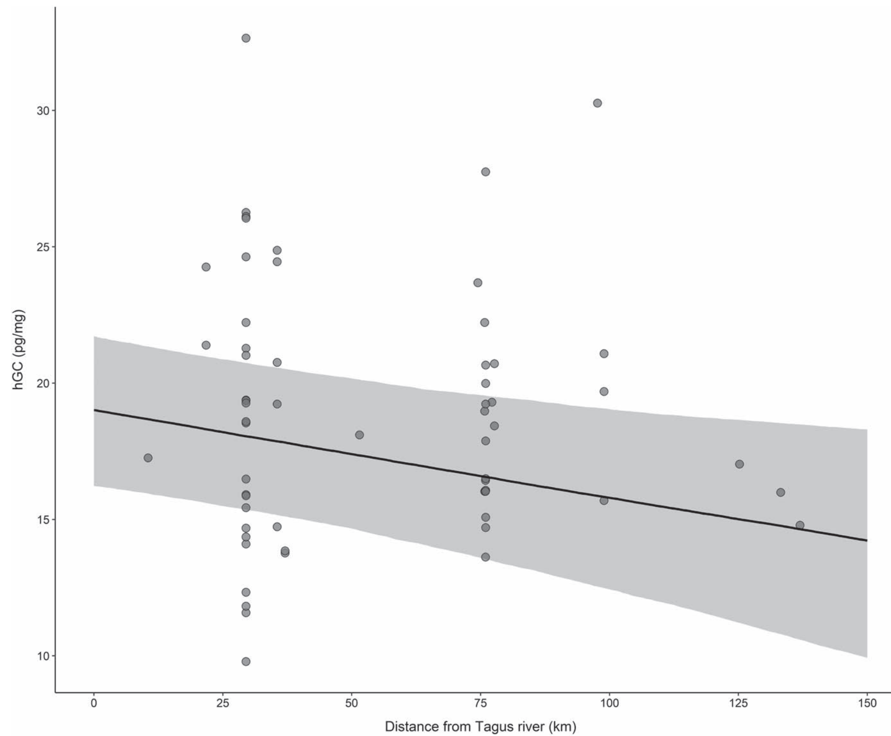

Figure 2: Model predictions of the effect of distance from the historic range (limited by the Tagus River) on hGC levels in the Egyptian mongoose. The plot shows model predictions and 95% CI (grey band) based on parametric bootstrapping with 5000 iterations.

distance from the Tagus River (and historic distribution) increased, which would equate to an estimated 1.0 pg/mg decrease in hGC every 32.26 km (Fig. 2). The estimated decrease in hGCs from the Tagus River to the expansion front (136.95 km) equates to 22% (4.24 pg/mg) of the mean hGC levels for the population (18.98 ± 5.42 pg/mg). These values were obtained while accounting for hGC variation with age, sex, season, sample storage time, body condition (BCS), size (STL) and environmental factors (conspecific and prey availability, human population density and relative area of favourable habitat). We found no evidence to support an effect of region (expansion vs. historic) on hGC levels. The effect of the distance to the Tagus River is influenced by three samples collected at distances above 125 km (Fig. 2), which, in case they were somehow related, would raise concern about possible confounding effects. Inspection of the data on the three samples revealed they were collected at different locations, in different years and seasons, by different people. Additionally, the effect of the distance to the historic region in an analysis performed without these three specimens resulted in qualitatively similar results.

It is not possible to discern whether our results reflect a phenotypic difference in long-term GC levels facilitating expansion or reduced GC exposure due to an environment with less frequent or severe stressors we could not account for (e.g. predators or competing carnivores). Decreasing GCs towards the expansion edge could reflect lower energy requirements to maintain physiological balance (McEwen and Wingfield, 2003), individuals in better condition and facing less challenges, potentially resulting in increased fitness (Bonier *et al.*, 2009a) or a reduced likelihood to exceed the normal and non-pathological response to environmental challenges (Romero *et al.*, 2009). The decreasing hGC levels towards the expansion front suggest that the Egyptian mongooses' expansion is not likely to be limited by mechanisms related to or mediated by the physiological stress response. The negative association between hGC and distance to the historic range is consistent with the colonization of a new area where stressors are less frequent or less severe (e.g. niche opportunity or forced dispersal), as well as a scenario where lower baseline GC levels favour dispersal or survival at the expansion front.

Our results apparently contradict the findings linking increased stress-induced GCs with expansion in house sparrows (Liebl and Martin, 2012). However, while stress-induced GC measurements in sparrows reflect the reactivity of the HPA-axis, long-term hGC levels are thought to reflect circulating GCs over several weeks. The latter method is inadequate to assess phenotypic differences in HPA-axis reactivity favouring dispersal and survival in novel environments. However, it is likely to provide a better indicator of chronic GC exposure, which is thought to

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Table 3: Relative importance of predictors based on the sum of Akaike weights in the complete set of 6656 candidate models

	Sum of weights	N containing models
STL	1.00	4608
Sex	0.99	4096
Age	0.98	4096
BCS	0.84	3328
Mongoose	0.72	3328
D. river	0.69	3328
Storage	0.63	3328
Season	0.61	3328
Rabbit	0.60	3328
Population	0.52	3328
Sex:STL	0.48	1536
Region	0.34	3328
Habitat	0.29	3328
Age:STL	0.13	1536

Legend: BCS, body condition score; STL, snout–tail length; Age:STL, interaction between age and snout–tail length; D. river, distance to the Tagus River for animals captured in the expansion region.

reflect the extent of environmental challenge to homeostasis (McEwen and Wingfield, 2003; Romero *et al*., 2009) and to potentially influence fitness through the effects of chronically elevated GC levels on most peripheral tissues (Sapolsky *et al*., 2000). Different response patterns and trade-offs between the effects of baseline and stress-induced GCs on fitness could explain simultaneously low baseline and increased stress-induced GC in expanding populations (Vitousek *et al*., 2018, 2019a). Due to the existence of a (albeit dynamic) threshold above which GCs cause deleterious effects to the organism, it is expected that lower baseline levels are required to allow higher levels of stress-induced increases without reaching pathological levels of GC exposure.

Body size (STL), sex, age and body condition (BCS) were included in all of the 16 models with ΔAICc $\leq$ 2.0, providing strong support for their inclusion in the model explaining hGC variation. Based on the sum of Akaike weights, STL had the highest relative importance (1.00), followed by sex (0.99), age (0.98) and finally BCS (0.84). Regarding the effect of sex on hGCs, the results of this study are consistent with our prior research, with males presenting higher GC levels compared to females (Azevedo *et al*., 2019). However, the variation of hGC among age cohorts is strikingly different from our previous work, where only type 1 juveniles differed from other cohorts, exhibiting the highest hGC levels. Here, with the inclusion variables reflecting body size and BCS, adults presented the highest hGC levels, followed by sub-adults, type 1 juveniles and finally type 2 juveniles. These results suggest that increased GC levels observed in juveniles could be at least partially driven by metabolic scaling or energy availability rather than exclusively by ontogenetic variation in endocrine mechanisms. Body size received the most support for inclusion in the model and had the strongest effect (−5.20, 95% CI: −7.56, −2.84) on hGCs that decreased with size. Energy reserves represented by BCS also received strong support for inclusion in the model and had a strong effect (−1.76, 95% CI: −3.09, −0.43), with hGC levels decreasing as body condition increased. Overall, the effects of variables accounting for normal patterns of hGC variation in this population were quite strong (with magnitudes ranging from 1.76 to 5.20) compared to the effect of spatial and environmental factors. Additionally, storage time was present in 14 of the 16 models with ΔAICc $\leq$ 2.0, with a sum of Akaike weights of 0.63 and a standardized effect of −3.65 (95% CI: −5.40, −1.90), supporting its inclusion in the model. These results illustrate how failing to include known causes of GC variation could confound the results of studies aiming to investigate the effects of environmental or spatial factors.

Human population density was expected to be associated with increased hGCs. However, we did not find strong support for its inclusion in the model (present in 11 of 16 models with ΔAICc $\leq$ 2.0, *sw* = 0.52 and 95% CI: −0.18, 2.62), questioning whether the species is severely stressed by human presence. Resilience to stress caused by human presence due to the species' known behavioural plasticity (Monterroso *et al*., 2014; Streicher *et al*., 2020) could have facilitated expansion in spite of increasing human population density. Alternatively, an attenuation of the stress response due to habituation (Cyr and Romero, 2009; Dickens and Romero, 2013) could explain absence of an increase in hGC levels with human density. Nevertheless, the result is discordant with the general trend in vertebrates, where an increase in GC levels is usually observed with increasing human disturbance (Dantzer *et al*., 2014). In the specific case of the Egyptian mongoose, presence–absence data previously revealed a negative influence of urban areas and human infrastructure on the species' occurrence (Barros *et al*., 2015). However, the absolute values of human density in our data were quite low, with a mean (and inter-quartile range) of human population data of 1(1–44) inhabitants per km^2, compared to the country's average of 112.5 (0–5244.6) inhabitants per km^2 (from Eurostat, European Commission, 2015) Therefore, although our results suggest little influence of human presence on Egyptian mongoose hGCs at these human population densities, an effect might be present at higher densities, warranting cautious interpretation of these results.

European rabbit and Egyptian mongoose harvest data were included in our model as proxies of relative prey availability and relative conspecific abundance, respectively. We expected hGC to decrease with relative prey availability and to increase with conspecific density. However, our results showed the opposite relation in both cases, with hGC increasing with prey availability and decreasing with mongoose abundance. The European rabbit is the Egyptian mongoose's main prey species, accounting for 28% of ingested biomass in

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Table 4: Model averaging results presented as estimates and 95% confidence intervals

	Standardized variables			Untransformed variables		
Factor	Estimate	95% Confidence interval		Estimate	95% Confidence interval	
Intercept	20.26	18.20	22.33	58.68	41.86	75.50
Age (juvenile 1)	−2.39	−5.89	1.11	−2.39	−5.89	1.11
Age (juvenile 2)	**−4.23**	**−6.56**	**−1.90**	**−4.23**	**−6.56**	**−1.90**
Age (Sub-adult)	−1.91	−3.99	0.17	−1.91	−3.99	0.17
Sex (male)	**2.08**	**0.88**	**3.28**	5.14	−4.25	14.53
Season (spring)	1.24	−0.73	3.22	1.24	−0.73	3.22
Season (summer)	−1.43	−3.31	0.45	−1.43	−3.31	0.45
Season (winter)	0.92	−1.39	3.22	0.92	−1.39	3.22
Mongoose	**−1.35**	**−2.69**	**−0.01**	**−0.26**	**−0.51**	**$-2.3e^{-3}$**
Rabbit	1.19	−0.05	2.42	0.01	$-4.0e^{-4}$	0.02
Body condition (BCS)	**−1.76**	**−3.09**	**−0.43**	**$-2.8e^{-3}$**	**$-4.9e^{-3}$**	**$-7.0e^{-4}$**
Human population	1.22	−0.18	2.62	0.01	$-9.0e^{-4}$	0.01
Body size (STL)	**−5.20**	**−7.56**	**−2.84**	**−0.21**	**−0.34**	**−0.09**
Storage	**−3.65**	**−5.40**	**−1.90**	**−0.01**	**−0.01**	**$-4.3e^{-3}$**
Distance to Tagus River	**−1.76**	**−3.26**	**−0.26**	**−0.03**	**−0.06**	**$-4.5e^{-3}$**
Sex × body size (STL)	−1.74	−4.10	0.62	−0.08	−0.18	0.03
Region (historic)	−0.78	−3.28	1.72	−0.78	−3.28	1.72

Legend: estimates are presented for model averaging using input variables standardized on two standard deviations following Gelman (2008) and using untransformed input variables. Confidence intervals not including zero are highlighted in bold as variables are considered to significantly influence hGC concentration.

this specific population (Bandeira *et al.*, 2018). Food scarcity has been associated with increases in GCs in some species (Bryan *et al.*, 2013, 2014; Riechert *et al.*, 2014; Barrett *et al.*, 2015) but in others no effect was detected (Van Meter *et al.*, 2009; Rector *et al.*, 2012; Burstahler *et al.*, 2019).

An equally surprising result was the negative effect of relative mongoose abundance on hGC. It is not clear whether high population density always leads to an increased stress response. Studies linking the GCs to conspecific density and intraspecific competition in aquatic (Leatherland, 1993; Glennemeier and Denver, 2002; Bolasina *et al.*, 2006; Ramsay *et al.*, 2006; Teixeira *et al.*, 2012) and social (Hawley *et al.*, 2006; Eggermann *et al.*, 2013) species have shown inconsistent trends. In a non-social and aggressively territorial species like the Egyptian mongoose (Palomares and Delibes, 1993), the increased frequency of antagonistic social interactions is expected to result in increased HPA-axis activity at high population densities (Creel *et al.*, 2013), especially during the breeding season. While a possible explanation is that aggressive interactions driven by territorial behaviour cause only transient stress responses that might not be reflected in hGC, the limitations of our harvest data on these results cannot be ignored. Firstly, hunting yields might be more reflective of removal of individuals than indicators of abundance. Lower hGC levels could be a consequence of the continuous mongoose removal through hunting, potentially alleviating territorial competition. Secondly, hunting activities can cause stress (Bryan *et al.*, 2015; Vilela *et al.*, 2020). The increasing number of rabbits removed by hunting activities could lead to an increased stress response in mongooses in those areas either by acting as competition for resources or by causing direct disturbance to mongoose. Rabbit hunting often involves the use of firearms and hunting dogs, while mongoose captures are usually undertaken by trapping (Ministério da Agricultura, Desenvolvimento Rural e Pescas, 2000). This difference in hunting methods could explain an increase in hCG with increasing rabbit harvest numbers. Finally, hunting data have no correction for sampling effort, which can bias abundance estimations. Even when a catch-per-unit-effort metric is used, catch data may overestimate abundance (Harley *et al.*, 2001). In the specific case of the rabbit, discrepancies have been reported between abundance estimates using hunting data and field data (Ferreira *et al.*, 2010). Therefore, while harvest data might provide a good metric for the assessment of the physiological impact of hunting activities on the Egyptian mongoose, caution is necessary when interpreting them as indicators of abundance.

We found no support for our prediction that larger areas of Mediterranean shrub and agro-forestry habitats within each grid cell would be associated with lower levels of hGCs. This

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result could be due to the mongooses' behavioural and dietary plasticity or alternatively by the dependence on resource-rich favourable habitat in the core areas of its territory that is not necessarily proportional to the size of the home range of each individual (Streicher *et al.*, 2020) or the area of favourable habitat available in each grid cell. Animal movement and high-resolution landscape data would be required to further analyse the relation between hGC and favourable habitat in the species.

Conclusion

In the Anthropocene, species distributions are changing at an unprecedented rate. A small number of wild species have expanded their ranges, contradicting the general trend of decline. The study of these exceptions can help predict future species distributions. This study is the first to examine the relation between GCs and range expansion in mammals and uses a long-term measure of GC levels that is less subject to the short-term influence of environmental variables. The results show a decrease in hGC levels towards the expansion front, suggesting that the species' expansion is unlikely to be limited by mechanisms related to- or mediated by the physiological stress response.

Supplementary material

Supplementary material is available at *Conservation Physiology* online.

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6. Discussion

Glucocorticoids have been measured in many types of samples in order to quantify stress in animals (Sheriff *et al.*, 2011a), but over the past twenty years, their measurement in hair has emerged as a promising tool to measure chronic physiological stress in wildlife. Using hair as a matrix allows the use of non-invasive sampling methods and provides an integrated value of circulating glucocorticoids over several weeks, that is not confounded by acute stress responses or sampling (Meyer and Novak, 2012; Russell *et al.*, 2012; Stalder and Kirschbaum, 2012), all of which are important advantages when working with wild and endangered species. However, rigorous and extensive species-specific validation is required in order to obtain meaningful results. In the current work, hair cortisol measurement by EIA is validated for two species of Iberian carnivores: the endangered Iberian lynx and the expanding Egyptian mongoose. Cross-validation using HPLC was performed to identify the compounds to which the assay antibodies were binding and next, EIA results were compared to gold standard (HP)LC-MS/MS measurements. After validation, normative patterns of variation with age, sex and season were determined, thus enabling to control for the effect of these factors when using hGCs as indicators of stress. The results of this validation are critically analysed in relation to major outstanding questions of hair glucocorticoid incorporation and metabolism. Finally, a practical example of the use of hGC measurement to understand animals' response to environmental change is provided in the form of a country-wide spatial analysis of hGC variation in the Egyptian mongoose.

6.1. Validation of EIA for hair cortisol measurement

Glucocorticoids have been measured in hair of many wild species over the past two decades (e.g. Koren *et al.*, 2002; Davenport *et al.*, 2006; Accorsi *et al.*, 2008; Macbeth *et al.*, 2010; Terwissen *et al.*, 2013; Carlitz *et al.*, 2014). In mammals it is well established that hair glucocorticoid measurements reflect long term increases in HPA-axis activity, as induced by repeated (del Rosario *et al.*, 2011; Terwissen *et al.*, 2013; Mastromonaco *et al.*, 2014; Endo *et al.*, 2018), but not one (Ashley *et al.*, 2011) or two (Tallo-Parra *et al.*, 2018) ACTH injections. This relation is further supported by the correlation observed between hair cortisol measurements and repeated measures of cortisol in plasma (e.g. Davenport *et al.*,

2006) and glucocorticoid metabolites in faeces (e.g. Mastromonaco *et al.*, 2014), as well as radio-metabolism studies (Kapoor *et al.*, 2018). Biologically active cortisol that is unbound to corticosteroid binding globulin is thought to passively diffuse into hair from blood capillaries or surrounding tissues, in what is known as the multi-compartment model (Henderson, 1993; Pragst and Balikova, 2006). However, many knowledge gaps persist regarding local production and glucocorticoid incorporation into hair (Ito *et al.*, 2005; Slominski *et al.*, 2007; Sharpley *et al.*, 2011; Keckeis *et al.*, 2012; Kapoor *et al.*, 2018), as well as the influence of external factors (Macbeth *et al.*, 2010; Cattet *et al.*, 2014; Salaberger *et al.*, 2016) on hair glucocorticoid concentrations. Additionally, trends in hair cortisol variation with factors related to sample characteristics (anatomical site, hair colour and hair length) and life-history stages (age, sex and reproductive season) have been inconsistent across species (Heimbürge *et al.*, 2019). Furthermore, prior experience with glucocorticoid measurement in other matrices shows that species differences in glucocorticoid metabolism and the influence of other compounds in the sample can confound assay results (Touma and Palme, 2005; Sheriff *et al.*, 2011a). Hence, species-specific validation is required in each case in order to obtain meaningful results (Touma and Palme, 2005; Sheriff *et al.*, 2011a; Article 2). This section discusses the validation process for hair glucocorticoid analyses in the Iberian lynx and the Egyptian mongoose.

6.1.1. Sampling, storage and extraction

A standard sampling location was defined for our study species in order to control for the effect of anatomical location on hair glucocorticoid levels (Burnard *et al.*, 2017). In the Egyptian mongoose (articles 1, 2 and 4), hair samples were clipped as close to the skin as possible from a standard anatomical location between the *scapulae*. In Iberian lynx, hair samples were collected by clipping the full length of hair from the inner thigh (articles 2 and 3). Anatomical location of the sample has been shown to influence hair cortisol measurements in many wild (Ashley *et al.*, 2011; Terwissen *et al.*, 2013; Yamanashi *et al.*, 2013; Carlitz *et al.*, 2015; Sotohira *et al.*, 2017; Acker *et al.*, 2018) and domestic (Moya *et al.*, 2013; Burnett *et al.*, 2014; Casal *et al.*, 2017; Duran *et al.*, 2017) species, but not in others (Comin *et al.*, 2012b; Macbeth *et al.*, 2012; Carlsson *et al.*, 2016; Schell *et al.*, 2017). Hair growth takes place during the anagen phase, followed by a regression phase (catagen) and a resting phase (telogen) (Dry, 1926), the duration

of which can vary between hair types (guard hairs or undercoat) (Ling, 1970). Based on the generally accepted assumption that cortisol passively diffuses into hair follicles during growth, this would only occur during anagen (Meyer and Novak, 2012). However, any hair sample will contain different relative amounts of hairs along the continuum spanning these phases, potentially affecting hair glucocorticoid measurements (Burnard *et al.*, 2017). Patterns of hair growth and moult can also differ between species and between seasons in the same species (Ling, 1970; Zimova *et al.*, 2018), which could induce further differences in proportions of anagen hairs sampled from different anatomical regions. A third dimension of variation is related with annual hair cycles. Temperate terrestrial mammals such as the Egyptian mongoose and the Iberian lynx moult twice a year, but detailed studies on the timing and duration of the moults are lacking, as for most species (Ling, 1970). The standardized collection of hair samples from the inter-scapular region of Egyptian mongoose was chosen to minimize confounding due to anatomical location, as well as faecal, urinary or salivary contamination. The inner thigh was chosen in the Iberian lynx to take advantage of hair clipped for blood collection from the saphenous vein during routine medical examinations of the captive breeding programme. The variation in hair patterns due to moults could not be accounted for in hair sampling protocols. Therefore, a potential influence of these mechanisms on seasonal variations of hair cortisol levels must be considered, because the timing of moults is determined by photoperiod (Ling, 1970). All Iberian lynx were sampled during the same period from November to December, effectively controlling for seasonal variation. In the case of the Egyptian mongoose, the month or season of sample collection were included in the analyses, but interestingly the effect of season was not significant as an explanatory variable for hair glucocorticoid variation, despite the availability of samples from every month of the year (see detailed discussion of the effect of season in section 3.2.3).

In Iberian lynx samples, poor repeatability was observed in duplicate and triplicate extracts produced from the same hair sample, resulting in glucocorticoid measurements that varied more than 40% (article 3). Iberian lynx coats display striking patterns of black markings against a light brown or orange background. Hence, the observed effect could be due to inadvertently taking different proportions of hair of each colour or hair growth phase from the larger hair sample. Intra-individual variation, with dark hairs containing higher cortisol

levels has been observed in some studies (Bennett and Hayssen, 2010; Yamanashi *et al.*, 2013; Burnett *et al.*, 2014) and lower levels in others (Macbeth *et al.*, 2010; del Rosario *et al.*, 2011; Tallo-Parra *et al.*, 2015; Hayashi *et al.*, 2021). Additionally, a small number of studies have failed to detect any difference (Nedić *et al.*, 2017; Nejad *et al.*, 2017). In order to control for this variation, an extra preparation step was added to the protocol, consisting of cutting and homogenizing the entire hair sample before sub-sampling for extraction (article 3). The extra step successfully improved the repeatability to within acceptable limits, and therefore presents a solution to control for the effect of intra-sample variation. To the authors' knowledge, the results reported in article 3 are the first to assess intra-sample variation in hair samples from coats with patterns, with implications for validation in new species of wildlife.

Full length hair samples from both study species were used in order to avoid confounding by sampling different lengths of hair. A high correlation was observed between measurements of five steroids in proximal and distal segments of Iberian lynx hair samples (article 3), which is consistent with prior findings in mammals (Bennett and Hayssen, 2010; Macbeth *et al.*, 2010; Yamanashi *et al.*, 2013; Carlitz *et al.*, 2014). These results indicate that further refinement is necessary to pinpoint the time of exposure and create chronological series of responses to stressors, but also suggest that using full length hair samples is unlikely to confound studies focusing physiological stress at a macro scale, such as those assessing species or cross-species responses to environmental disturbance (e.g. Jessop *et al.*, 2013; Vitousek *et al.*, 2019a).

An effect of storage was found on Egyptian mongoose hair samples, with cortisol levels declining as storage time increased, after accounting for temporal variation (article 1). Cortisol has been demonstrated to remain stable in intact hair samples stored for periods of up to 17 months at room temperature, and for shorter periods in refrigeration or frozen, but in powdered samples declines were noted as early as the first month (Burnard *et al.*, 2017). Egyptian mongoose specimens were kept frozen at -20ºC from 2.5 to 6.2 years, largely exceeding storage times in prior studies. The observed effect of long-term storage on Egyptian mongoose hair cortisol was strong when compared to spatial and environmental variables (article 4), which demonstrates the need to control for storage time when measuring glucocorticoids in hair specimens stored for long periods. With the

increasing use of glucocorticoid measurements from matrices with long-term stability, accounting for storage should be given consideration, especially when using samples obtained from biobanks or museum specimens.

6.1.2. HPLC cross-validation: what are EIAs binding to?

HPLC immunograms revealed that cross-reactivity of the cortisol-EIA antibodies with several compounds in hair extracts was present in both species (articles 1, 2 and 3). Measurements with the cortisol-21-HS EIA were eight times higher than with the cortisol-3CMO EIA in the Egyptian mongoose and two times higher in the Iberian lynx (article 2). Both EIAs co-eluted with peaks of cortisol and cortisone standards, but the cortisol-21-HS EIA cross-reacted with several unknown compounds in both species (article 2, figure 3). Although common practice when validating methods for faecal glucocorticoid metabolite testing (Möstl and Palme, 2002), HPLC immunograms have seldom been used to validate methods of hair glucocorticoid measurement. In guinea pigs (*Cavia aperea f. porcellus*) the results were similar, with cortisone co-eluting with corticosterone in the cortisone EIA, and cortisol co-eluting with cortisone in the cortisol EIA, and cross-reactivity with several unknown compounds present in the hair extracts in both EIAs. Salaberger *et al.* (2016) observed high specificity for cortisol using both cortisol-21-HS EIA and cortisol-3CMO EIA in sheep hair samples that were subject to a brushing treatment. However, in samples subjected to a local dexamethasone treatment, the authors found contrasting results, with no peak in the cortisol fraction and cross-reactivity with several unknown compounds. In article 2 of this work, cross-reactivity profiles of the cortisol-21-HS and cortisol-3CMO EIAs were shown to vary between six species, which could potentially explain the differences found in guinea pigs and sheep, and cautions against extrapolation from one species to another (Article 2). High levels of antibody cross-reactivity lead to a lack of specificity that could potentially hinder the EIA's use. In the Egyptian mongoose, cortisol and cortisone together accounted for 10.5% of the total immunoreactivity of the cortisol-21-HS EIA (article 2). The potential for error due to this effect was illustrated by simulating the model construction used in article 1 (with cortisol-3CMO EIA data) using the cortisol-21-HS EIA data. In the simulation, the model results using the cortisol-21-HS EIA data were strikingly different from the cortisol-3CMO EIA model, and were obscuring the effect of sex and storage time on hair glucocorticoid measurements (article 2, table 2). These results provide a

practical example of how choosing the wrong EIA can lead to erroneous results, and reiterate the need for rigorous validation of EIAs to measure hair glucocorticoids in new species. For this purpose, the current work shows that HPLC immunograms provide a valuable tool to determine the specificity of EIAs targeting steroids in hair.

6.1.3. LC-MS/MS cross-validation: what is really in the hair samples?

Cortisol-21-HS and cortisol-3CMO EIAs overestimated steroid concentrations in hair extracts when compared to (gold standard) LC-MS/MS measurements (article 2, table 3). The comparison between the results obtained by LC-MS/MS and EIA was consistent with the findings of the HPLC analysis. In the mongoose, two- and ten-fold overestimations of cortisol were observed with the cortisol-3CMO and cortisol-21-HS EIAs, respectively. In the Iberian lynx, cortisol-3CMO EIA values matched the cortisol + cortisone LC-MS/MS results, but the cortisol-21-HS resulted in a two-fold overestimation. Lynx cortisol-3CMO EIA measurements were correlated with the sum of cortisone and cortisol measured by LC-MS/MS, thus supporting the HPLC findings of antibody cross-reactivity. In an analogous approach using human hair, Slominski *et al.* (2015) found LC-MS/MS cortisol measurements to be lower than- and correlated with those obtained by their EIA. The authors attributed the difference to a higher sensitivity of the EIA. However, cortisone was also detected by LC-MS/MS in their extracts, and therefore overestimation due to EIA cross-reactivity could be an alternative explanation for their results. Findings in the present work illustrate to what extent EIAs can overestimate hair glucocorticoid measurements due to cross-reactivity with cortisone and with unknown compounds, and emphasize the importance of EIA validation prior to application.

LC-MS/MS measurements also revealed that cortisol and cortisone varied in different directions when comparing male and female Iberian lynx (article 3, figure 5). The differences in cortisone were consistent with higher male hair cortisone levels observed in humans (Raul *et al.*, 2004; Stalder *et al.*, 2013) but not in sheep (Stubsjøen *et al.*, 2015). Opposite directions of cortisone and cortisol variation can potentiate the confounding effect of EIA cross-reactivity that was present in both our study species (articles 1, 2 and 3), as well as other studies (Keckeis *et al.*, 2012; Salaberger *et al.*, 2016). For example, in Iberian lynx hair extracts, cortisol measured by LC-MS/MS was significantly higher in females

than in males (article 3). However this difference was obscured in the cortisol-3CMO EIA results, at least partially due to the EIA cross-reactivity with cortisone, which showed an opposite direction of variation with sex in LC-MS/MS results (article 3, figure 5).

Captive born and wild caught Iberian lynx also exhibited opposite trends in LC-MS/MS measurements of hair cortisol and cortisone (article 3, figures 6). As expected, captive-born animals of the breeding programme had lower cortisol levels than their wild-caught counterparts, but they had higher cortisone levels. This suggests a stress-induced variation of the two steroids in opposite directions. Cortisone levels have been shown to correlate with cortisol in saliva, blood and urine (Morineau *et al.*, 1997; Perogamvros *et al.*, 2010), as well as hair (Stalder *et al.*, 2013; Staufenbiel *et al.*, 2015), contradicting these results in Iberian lynx. This has led the suggestion of cortisone as an alternative indicator of HPA-axis activity (Perogamvros *et al.*, 2010; Stalder *et al.*, 2013; Zhang *et al.*, 2017). Stalder et al. (2013) found hair cortisol and cortisone to co-vary in humans diagnosed with metabolic syndrome and in relation to cardiometabolic indicators. However, in the same study, the authors noted a group of cortisone-cortisol data pairs with disproportionately high hair cortisol levels. Conversely, a decrease in serum cortisone levels has been demonstrated while cortisol increases in response to ACTH stimulation (Vogeser *et al.*, 2001), during illness (Vogeser *et al.*, 2002) and after surgery (Vogeser *et al.*, 2003). This raises the question of whether the unexplained cases observed by Stalder et al. (2013) and the differences between captive born and wild caught Iberian lynx could be justified by stress-induced variation of cortisol and cortisone in opposite directions. Unfortunately, the small sample size and sex bias in the groups of Iberian lynx limits interpretation. The only confirmed case of a life-threatening and prolonged stressor available in the present work is that of an Iberian lynx that escaped during a wildfire that led to the evacuation of the breeding centre, and was captured 24 days later. A longitudinal series of EIA measurements revealed a decrease in hair cortisone and an increase in cortisol 3 weeks after recapture, that had subsided a year later (article 3, figure 3). These results are consistent with the findings of Vogeser *et al.* (2001, 2002, 2003) and support a stress-induced variation of cortisol and cortisone in opposite directions.

One possible explanation for opposite trends in cortisol and cortisone variation is the interconversion of cortisol and cortisone (or corticosterone and 11-dehydrocorticosterone) catalysed by 11β-hydroxysteroid dehydrogenase (11β-HSD) isoenzymes 1 and 2. This interconversion mechanism regulates the ratios of active (cortisol or corticosterone) and inactive (cortisone or 11-dehydrocorticosterone) glucocorticoids, allowing concentrations in peripheral tissues to be different from circulating levels (Roland and Funder, 1996; Jamieson *et al.*, 1999) and regulating the concentrations of active glucocorticoids available to bind with glucocorticoid and mineralocorticoid receptors (Seckl and Walker, 2001; Tomlinson and Stewart, 2001; Wyrwoll *et al.*, 2011). Therefore, glucocorticoid action on target tissues is determined by intracellular pre-receptor 11β-HSD activity, in addition to circulating hormone levels, introducing an additional level of HPA-axis regulation (Harris *et al.*, 2001). In humans, 11β-HSD1 is present in the epidermis and 11β-HSD2 is expressed in sweat glands (Hirasawa *et al.*, 1997; Tomlinson and Stewart, 2001), suggesting it may be present in tissues adjacent to hair follicles in other mammals. Indeed, Raul *et al.* (2004) proposed this mechanism as an explanation for the presence of cortisone in hair samples. More recent results of radio metabolism studies clearly demonstrate that radiolabelled cortisol is found in hair in the form of radiolabelled cortisone in both guinea pigs (Keckeis *et al.*, 2012) and primates (Kapoor *et al.*, 2018), thus challenging the generally accepted theory of passive incorporation from surrounding tissues (Henderson, 1993; Pragst and Balikova, 2006) and lending additional support to the hypothesis of conversion to cortisone before, during or after incorporation into hair. Since free cortisone in circulation does not appear to increase in response to ACTH stimulation (Vogeser *et al.*, 2001; Perogamvros *et al.*, 2010), conversion is likely to take place locally, during or after glucocorticoid incorporation into cells of peripheral tissues. Hair glucocorticoid increase has been demonstrated in response to local stressors (Sharpley *et al.*, 2011) and to CRH stimulation of hair follicles (Ito *et al.*, 2005; Lee *et al.*, 2020), which has led to the proposition of a local HPA-axis analogue. As 11β-HSDs in skin adipocytes have been shown to be subject to modulation by CRH and ACTH (Friedberg *et al.*, 2003), the existence of such a mechanism in hair follicles could mimic the response of the HPA-axis and partially justify local responses. It is currently unknown whether 11β-HSDs are expressed in hair follicles and whether local production of cortisol is due to conversion from cortisone catalysed by these

enzymes, an alternative pathway, or both. Hair glucocorticoid metabolism seems to be surprisingly more complex than previously thought. It is important to disentangle to what extent glucocorticoid variation in hair reflects passive incorporation of circulating hormones, local 11β-HSD interconversion or production by a local HPA-axis analogue in order to obtain more robust and consistent indicators of physiological stress. Expanding on the approach used in this work by simultaneously measuring cortisol and cortisone in hair in response to a repeated ACTH challenge, as well as 11β-HSD expression hair follicles and surrounding tissues, could help clarify these questions.

DHEA was the most abundant steroid measured in Iberian lynx hair by LC-MS/MS (article 3). Cortisol-cortisone and cortisol-DHEA ratios were significantly different between captive-born and wild-caught Iberian lynx held in captivity (article 3, table 1 and figure 6), while cortisol and cortisone alone showed only trends in opposing directions. DHEA has been consistently observed in higher concentrations than cortisol measured by radio immunoassay in pigs (Bergamin *et al.*, 2019) and dairy cows (Peric *et al.*, 2017), and by LC-MS/MS in humans (Gao *et al.*, 2013; Qiao *et al.*, 2017). Due to the anti-glucocorticoid effects of DHEA, its competitive inhibition of cortisone binding to 11β-HSD1 (Hennebert *et al.*, 2007) and regulation of 11β-HSD1 and 11β-HSD2 expression, DHEA levels and cortisol-DHEA ratios have been proposed as indicators of resilience to stress and stress-related pathology (Maninger *et al.*, 2009). Higher serum and urine cortisol-DHEA ratios have been associated with stress, anxiety and depression, although not consistently (Maninger *et al.*, 2009), but it is difficult to exclude the confounding effect of diurnal fluctuations in serum hormone levels. In humans with HIV, highly stressed individuals showed increased hair cortisol and lower DHEA-cortisol ratio compared to the low-stress group, which was associated with perceived stress and stressful life events (Qiao *et al.*, 2017). In the present work, the small number of lynx hair samples analysed by LC-MS/MS and the sex bias between groups of captive-born and wild-caught animals limit the ability to distinguish whether the effects are due to sex or origin. Nonetheless, the fact that significant group differences were detected in cortisol-cortisone and cortisol-DHEA ratios in such a small sample (n=12) of Iberian lynx suggests there is potential in a more comprehensive approach to hair glucocorticoid measurement in wildlife. Studies focusing on several hair steroids (such as cortisone and DHEA) and their ratios in

relation to cortisol might help advance the understanding of local steroid metabolism and how hair steroid levels vary in response to stress.

6.2. Patterns of variation in hair glucocorticoids

Glucocorticoids are necessary for the normal function of organisms, and can be present at different levels depending on several factors. Hair glucocorticoids may undergo variations according to circadian rhythms, sex, seasonal cycles and life-history phases (section 1.3). Differences in glucocorticoid levels can also be expected due to energetic demand and metabolic rate (Sapolsky *et al.*, 2000). Many of these factors seem to inconsistently affect glucocorticoid levels across species, but accounting for all of them is seldom possible. The following section discusses the variation in hair glucocorticoid levels observed in Iberian lynx and Egyptian mongoose.

6.2.1. Sex

In the Egyptian mongoose, cortisol levels measured by cortisol-3CMO EIA were estimated to be higher in males, compared to females (article 1, table 2; article 4, table 4). This effect was present after accounting for the effect of age, month and season, storage time, environmental factors, body size and body condition. Inconsistent trends in hair cortisol have been identified across species, with males presenting higher (Lafferty *et al.*, 2015; Schell *et al.*, 2017), lower (Bechshøft *et al.*, 2011; Cattet *et al.*, 2014; Dettmer *et al.*, 2014) or similar (Terwissen *et al.*, 2013; Yamanashi *et al.*, 2013; Carlitz *et al.*, 2014) hair cortisol concentration, compared to females. Heimbürge *et al.* (2019) suggest that higher hair cortisol in males could be due to stressful behaviours related to reproduction or reduced 11-βHSD-2 enzyme activity. The Egyptian mongoose is highly territorial, and aggressive encounters are frequent between males (Palomares, 1991; Palomares and Delibes, 1993), which could explain elevated cortisol levels in males.

In the Iberian lynx, sex differences in cortisol obtained from analyses of EIA results conflicted with those obtained from LC-MS/MS measurements (article 3, figure 5). Based on measurements using cortisol-3CMO EIA, males had higher cortisol concentrations compared to females. However, LC-MS/MS measurements indicate higher levels of cortisol in females compared to males, coupled with an

inverse pattern in cortisone. Clarification can be obtained from the HPLC analysis (article 3, figure 4) that shows that cortisol-3CMO EIA antibody is binding to cortisone (30% immunoreactivity) at an extent similar to cortisol (36% immunoreactivity), which results in an overestimation of cortisol levels by EIA. This hypothesis was tested by examining the correlations between LC-MS/MS and cortisol-3CMO EIA measurements. The results showed that cortisol-3CMO EIA measurements were correlated with LC-MS/MS measurements of cortisol + cortisone and cortisone, but not cortisol alone (article 3). Since males have higher levels of (LC-MS/MS) cortisone compared to females, EIA cross-reactivity would result in a disproportionate overestimation of cortisol between sexes. These findings raise the question of whether overestimation due to EIA cross-reactivity to cortisone could explain the higher hair cortisol levels in Egyptian mongoose. While it is certainly a possibility, antibody binding to cortisone in the mongoose was lower (7% in article 1, figure 1; 11% in article 2, figure 3) than observed in Iberian lynx, making this explanation less likely. Nonetheless, LC-MS/MS results clearly demonstrate that hair cortisol levels were higher in Iberian lynx females than in males. This is consistent with the findings in pigs (Bergamin *et al.*, 2019) and female polar bears with dependant offspring (Macbeth *et al.*, 2012), and could indicate sex-specific HPA-axis activity to respond to specific conditions of reproductive life-history. It must be noted that all of the Iberian lynx samples are from pre-breeding season medical examinations between November and December. It is therefore unclear whether this trend will remain consistent throughout the year, or if sex-dependant seasonal variations related with reproductive cycles could influence glucocorticoid levels in the species (see section 3.2.3).

6.2.2. Age

Hair cortisol measured by cortisol-3CMO EIA varied with age in the Egyptian mongoose. In article 1 (table 2), the model accounted for age, sex, season, storage time, and month and year of sample collection, and estimated hair cortisol to be higher in juveniles between two-and-a-half and five-and-a-half months (juvenile 1), compared to all other age classes. In article 4 (table 4) the results were different, with juveniles between five-and-a-half and nine months (juvenile 2) presenting higher estimates cortisol-3CMO EIA measurements than juvenile 1, adults and sub-adults. The difference between the two results could be

due to inclusion of additional spatial, environmental and morphological factors in the analyses for article 4. Specifically two factors that were included in the candidate model in article 4 – body size and body condition – could partially explain the variation in hair glucocorticoids with age. An age-dependent decline in hair cortisol has been described in non-human primates (Laudenslager *et al.*, 2012; Dettmer *et al.*, 2014; Fourie *et al.*, 2016) and domestic herbivores (del Rosario *et al.*, 2011; Montillo *et al.*, 2014; Hayashi *et al.*, 2021; Comin *et al.*, 2012a). In two studies measuring hair cortisol in Grizzly bears (Macbeth *et al.*, 2010) and Canada lynx (Terwissen *et al.*, 2013), no effect of age was observed, but in polar bears results are inconsistent, with one study showing no effect (Bechshøft *et al.*, 2011) and another showing an increase in cortisol in young compared to adults in males only (Macbeth *et al.*, 2012). Further research is necessary to discern whether the juvenile hypercortisolism observed in the Egyptian mongoose is a result of body size, energy reserves or age-dependant differences in HPA-axis modulation, but the differences between the results with or without accounting for body condition and body size suggest that the difference could be partially modulated by effects of metabolic rate and energy demand. In the Iberian lynx we found no increase in cortisol-3CMO EIA measurements in juveniles compared to adults, and no effect of age on hair cortisol concentrations measured by LC-MS/MS (article 3). These results are not surprising given that all juveniles were already more than six months old, at full adult size and well beyond the post-weaning phase. Further research is required to assess if juvenile hypercortisolism also exists in the species.

6.2.3. Season

All Iberian lynx samples were collected from the same time of year, and therefore seasonal variations could not be assessed. In the Egyptian mongoose, there was no strong evidence of an effect of season on hair glucocorticoid measurements (articles 1 and 4). These findings are inconsistent with the general expectation of seasonal fluctuations in HPA-axis activity as a result of reproductive cycles and metabolic demands, based on the measurement of plasma cortisol and faecal glucocorticoid metabolites (Romero, 2002; Romero *et al.*, 2008). The effect of hair growth patterns could obscure the effect of season in hair glucocorticoid measurements due to the retention of non-growing hairs for period of up to a year, depending on the number of annual moults. In the mongoose, the absence

of an effect of the interaction between sex and season, and of reproductive state in females on cortisol-3CMO EIA measurements further supports the absence of seasonal variation in hair cortisol. However, while season did not significantly improve model performance in article 1, there was moderate support for its inclusion in the most parsimonious model in article 4, and in both studies trends suggested hair glucocorticoid concentrations might gradually increase from summer to winter, culminating with the highest levels in the breeding season in spring. Similar trends in hair cortisol concentrations have been reported in dogs (Roth *et al.*, 2016) and pigs (Bacci *et al.*, 2014), but different trends have been observed in other species such as bears (Cattet *et al.*, 2014) and chipmunks (Martin and Reale, 2008). A study in Vancouver Island marmots even found different trends in seasonal hair cortisol concentrations between different body regions (Acker *et al.*, 2018). When comparing the life-history of a bear to that of a mongoose, it is logical to expect different seasonal patterns of glucocorticoid levels due to the striking differences between the reproductive cycles and hibernation. Therefore, seasonal effects on hair glucocorticoid concentrations should always be accounted for when using hair glucocorticoids to measure physiological stress. Knowledge of moult and hair growth patterns can also improve the understanding of seasonal variation in hair glucocorticoids, but is lacking for most species.

6.2.4. Body size and body condition

We found evidence of an effect of body size and body condition score on hGC in wild Egyptian mongoose, with hGC levels decreasing with increases in both variables (article 4). Body size, represented by mongoose snout-to-tail length, was the variable with the highest relative importance and largest effect size of all the variables in the candidate model. It was therefore the most relevant variable in explaining variation in hGC levels in our data. Body condition score also had a high relative importance and a strong effect. These results are consistent with meta-analyses relating plasma glucocorticoid levels with body mass, but not necessarily mass-scaled metabolic rate (Haase *et al.*, 2016; Francis *et al.*, 2018). The presence of significant effects of both body size and body condition score suggest independent relations of hGC levels with metabolic rate and energetic reserves. These effects have important implications for practical applications. In article 4, the relative variable importance and effect strength of body size and

body condition on mongoose hGCs were higher than those of the spatial and environmental variables being researched. Therefore, not accounting for these two factors can easily confound the results. This seems to have been the case with the relation between mongoose age and hGCs that yielded different results before and after accounting for body size and body condition (article 4), thus suggesting that the relation was partly driven by body size or body condition. In field conditions, obtaining comprehensive data on study animals can be difficult, especially if they are free-ranging, and these variables are seldom controlled for. This was the case in our studies with Iberian lynx (article 3), where body size and body condition were not available. Although all animals in the study were all of adult size, it is not possible to exclude a confounding effect of size and body condition on the results. Our findings support careful consideration of the effects of body size and body condition in the design or interpretation of results of studies measuring hGCs in mammals.

6.3. Practical application of hair glucocorticoid measurement

Glucocorticoids influence animals' ability to respond to change in their environment (Wingfield *et al.*, 1998; Zimmer *et al.*, 2020), survive and reproduce (Sapolsky *et al.*, 2000), and are consequently expected to affect their *fitness* (Breuner *et al.*, 2008; Vitousek *et al.*, 2018b). Consequently, GC measurements have been extensively used as part of assessments of stress, welfare and adaptation to captivity in wild animals held in zoos or conservation programmes. Additionally, the use of glucocorticoid measurements to assess the status of populations of wild animals is also becoming increasingly popular, but the relations between GCs with stress (MacDougall-Shackleton *et al.*, 2019; Romero and Beattie, 2021) and fitness have been inconsistent (Bonier *et al.*, 2009). These inconsistencies could be due to the lability (Bonier *et al.*, 2009) and low intra-individual repeatability (Vitousek *et al.*, 2018b, 2019b) of point samples of plasma GC measurements, compared to samples that reflect longer periods of GC exposure (Taff *et al.*, 2018). Among the commonly used matrices to measure GCs, hair represents the longest period of exposure, and could help clarify these inconsistencies. Using hGC measurements, we assessed GC variation in Iberian lynx in captivity and Egyptian mongoose in the wild. In Iberian lynx, we tested for effects of behaviour (repetitive behaviour) and management (enclosure changes, institution transfers, enclosure area and captures) on hGC. In a wild population of

Egyptian mongoose, we assessed GC variation according to spatial and environmental factors in the context of range expansion.

6.3.1. Hair glucocorticoid variation in captive Iberian lynx

In Iberian lynx, we found no relation between behavioural and management variables and hGC measurements, but a longitudinal series of hGC measurements revealed an inversion of the cortisol-cortisone ratio in an individual escaped during a wildfire (article 3). Behavioural variables included were average daily frequency of repetitive behaviour for the month and the year prior to hair sampling. The management variables tested were breeding centre, origin, enclosure area, days since last- and total lifetime captures, enclosure changes and institution transfers. Our results are inconsistent with previous literature in which increases in faecal glucocorticoid metabolites (e.g. Liu *et al.*, 2006; Shepherdson *et al.*, 2013; Vaz *et al.*, 2017) and hair cortisol (Osman *et al.*, 2020) have been associated with higher frequencies of stereotypic behaviour in captive wild animals. Associations between hGC and capture (e.g. Cattet *et al.*, 2014) and enclosure change (e.g. Davenport *et al.*, 2006) or relocation (e.g. Yamanashi *et al.*, 2016) were expected, but not present in Iberian lynx (article 3). These results could be related to the confounding effect between variables, or with the mismatch between the temporal representation of the hGC measurement and the behaviour and management variables considered. The small sample size (n=19) limited the statistical analyses and made it unfeasible to perform multivariate analysis to account for the simultaneous influence of the several behavioural and management factors, and normative patterns of variation. In terms of temporal representation, while hGC are thought to reflect GC levels over the past few weeks, the days since last capture or institution transfer in our study exceeded six weeks and ten months, respectively, rendering the hair samples unrepresentative period of interest. It is fundamental that future research of the relations between behavioural and management variables and hGC in the Iberian lynx be designed to assure temporal representation of the GC measurements and consider confounding effects between variables.

Another approach that holds potential is the use of hGC measurements to create timelines of GC exposure. While variation along hair length has been the most frequent approach (e.g. Carlitz *et al.*, 2014), the non-invasiveness of hair sampling allows repeated sampling over time to create longitudinal hGC profiles.

Using this approach, an increase in cortisol was observed in an Iberian lynx in the weeks following escape during a wildfire. A decrease in cortisone was observed simultaneously, creating an inversion of the cortisol-cortisone ratio. Despite the important limitations associated with a sample of one animal, these results have important implications for measuring chronic changes in adrenocortical activity in captive animals. First, repeated sampling allows establishment of "baseline" values for each individual, effectively overcoming the problem of inter-individual variation in small samples. Second, longitudinal series of hair samples enable an objective temporal association of hGC values with candidate stressors, overcoming the uncertainty associated with knowledge gaps about GC distribution along the hair shaft. Longitudinal studies simultaneously measuring hair cortisol, cortisone and DHEA in response to ACTH challenge or known stressors are necessary to provide a more comprehensive understanding of how hGC relate to circulating GCs (detailed discussion in section 3.1.3.).

6.3.2. Hair glucocorticoid variation in wild Egyptian mongoose

In wild Egyptian mongoose, we found a decrease in hGCs toward the range expansion front, limited statistical evidence of an effect of human population or area of favourable habitat on GC levels, and counter-intuitive associations between hGCs and prey and conspecific abundance (article 4). Only four studies to date have assessed GC variation during range expansions (Atwell *et al.*, 2012; Liebl and Martin, 2012; Brown *et al.*, 2015; Martin *et al.*, 2018). All of them used baseline and stress-induced plasma corticosterone and none of them focused on mammals. The current work is the first to measure GC variation in a mammalian range expansion, and to use a matrix representing a long-term integrated measure of GCs. The decrease in hGC toward the expansion front in the Egyptian mongoose seems to conflict with the aforementioned studies. However, plasma GC measurements represent point samples of baseline circulating GCs and stress-induced HPA-axis reactivity, while hGC represent an integrated measure of total GC exposure over a period of weeks. Hence, while plasma GC measurements are useful indicators of phenotypic variations in the stress response, hGCs are expected to better represent allostatic load. Both approaches are therefore complementary, and future research combining them might help discern between GC variation in range expansions due to selection or facilitation by phenotypic

traits and GC variation due to the intensity and frequency of stressors in the colonized environment.

There was limited evidence supporting an effect of human population density on Egyptian mongoose hGCs, contradicting a consistent trend of GC increase in wild animals with human presence (Dantzer *et al.*, 2014). One possible explanation for this finding discussed in article 4 is habituation to human presence due to the behavioural plasticity of mongoose species. However, it could be that it is simply the result of disentangling the effects of other spatial and environmental factors and normative GC variation in the species from the effect of human presence. The absence of an effect of the available area of favourable habitat and counter-intuitive effects of indicators of relative prey availability and conspecific abundance were also inconsistent with general predictions. hGC levels were unaffected by the area of favourable habitat, decreased with reports of mongoose harvest, and increased with reports of rabbit harvest (article 4). Several explanations for these findings are explored in the article. For example, behavioural plasticity of the species could the lack of a relation between favourable habitat and GC, and differences hunting methods could justify the surprising relations of hGC with harvest data. While mongoose are usually trapped, rabbit hunting takes place with dogs and firearms, with the latter being more likely to elicit a stress response in wild animals in the vicinity. However, two important methodological considerations arise from these findings. Firstly, when using linear mixed effects models, the comparison of effect sizes (model coefficients) between independent variables is usually impaired by differences in the scale of the variables. In article 4, input variables were standardized (Gelman, 2008) to enable a better understanding of the factors influencing hGC variation, by allowing direct comparison between the effect sizes of each variable. This approach is likely to prove useful in studies exploring variation of physiological indicators in wild populations, where a large number of factors might be driving variation of physiological measures to different degrees. Secondly, all of the variables reflecting normative variation in GCs (age, sex, body size, body condition and storage) had higher relative variable importance and stronger effects than the environmental factors. This illustrates how easy it is to confound the assessment of the hGC variation with environmental variables by not accounting for normative patterns of GC variation. At least in this species, the results of studies exploring hGC variation in relation to any environmental

variable without accounting for normative GC variation in the species would almost certainly be confounded by their strong effect.

6.4. Conclusions and future perspectives

Hair glucocorticoid measurements have tremendous potential as non-invasive physiological indicators for wild mammals. In this work, EIAs were developed and validated for hGC measurement in two species of Iberian carnivorans, which have experienced opposing impacts from anthropogenic environmental change: the threatened Iberian lynx and the thriving Egyptian mongoose. The non-invasive techniques validated in this work will contribute to the understanding of stress physiology in both species and help identify GC patterns in captive and wild populations, thereby providing insights into how they respond to environmental change. In conservation programmes, hGC measurements can help identify factors influencing adaptation success and welfare in captivity and during reintroductions. In wild populations, GC measurement using samples from hair snares could provide information on factors causing physiological stress, and help identify areas where individuals are under higher pressure.

Prior validation of the EIAs using LC-MS/MS and/or HPLC is necessary to overcome limitations due to antibody cross-reactivity that can lead to erroneous results (articles 1, 2 and 3). Normative patterns of hGC variation were not consistent between study species (article 1, 3 and 4) and need to be accounted for. A critical assessment of the influence of age, sex, body size and body condition score on hGC variation in the Egyptian mongoose illustrated how easily failing to account for these factors can confound studies based on hGC measurement (article 4).

In terms of practical application, cortisol-cortisone and cortisol-DHEA ratios in Iberian lynx hair were more sensitive to differences between captive-bred and wild-caught individuals held in captivity, and an inversion of the cortisol-cortisone ratio was observed in an animal that escaped during a wildfire. These findings identify avenues for future research related with cortisol incorporation into hair, its interconversion with cortisone, and how different hGCs relate with circulating cortisol in response to stressors. The application of hGC measurement to wild animals was demonstrated by assessing spatial GC variation in Egyptian mongoose from the species' historic range to the expansion front, after EIA

validation and accounting for normative patterns of variation. A decrease in hGCs toward the expansion front was observed, which is consistent with predictions for a colonization of a favourable new area. Importantly, hGC data in the mongoose allowed the identification of factors that could have favoured the expansion, such as the apparent lack of an effect of human density on GC levels in this mongoose population.

There are some limitations that need to be considered when using hGCs as indicators of the physiological stress response. These limitations are related with assay methods (article 2), knowledge gaps in fundamental physiology (article 3) and interpretation of the results (articles 1, 3 and 4). Some of these limitations can be overcome by performing appropriate validation, characterizing normative patterns of variation, and/or considering them in study design and analyses. However, it remains unclear how and when cortisol is incorporated into hair, how it is converted into cortisone, and how hair cortisone and cortisol levels relate to stress-induced elevations in circulating cortisol. In the future, studies simultaneously measuring hair cortisol, cortisone and DHEA in response to ACTH injections or known stressors could help improve our understanding of local hGC metabolism. Studies characterizing the expression of 11β-HSD enzymes and glucocorticoid and mineralocorticoid receptors in hair follicles and surrounding tissues could also help clarify local GC pathways. Surprisingly, there is very little information on annual hair cycles and hair growth patterns in mammals, which will be necessary to refine the temporal representation of GC levels present in each hair sample. In cases like that of the Egyptian mongoose range expansion, it is not possible to distinguish based on hGC alone whether GC variation is due to phenotypic traits under selective pressure/favouring expansion, or due to differences in frequency or intensity of stressors in the novel environment. Future studies simultaneously measuring baseline and stress-induced plasma GCs and hGCs could help disentangle the causal mechanisms underlying these variations.

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