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REVIEW

Non-invasive measurement of glucocorticoids: The reptile perspective

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Abstract

Recent advancements in stress physiology, driven by the relevance of the stress response in animal welfare and conservation, have focused on alternative techniques beyond blood sampling for measuring glucocorticoids (GC). While blood samples have been traditionally used, practical and ethical concerns have spurred exploration into minimally invasive media like saliva, feces, milk, hair, and feathers. This review addresses the dearth of research on reptile endocrinology, offering insights into measuring GC or their metabolites in reptiles through various biological tissues. It underscores the importance of considering temporal dynamics in stress response evaluation and advocates for further exploration of alternative tools to enhance our understanding of reptilian stress responses.

Introduction

Animals are constantly confronted by a wide range of biotic and abiotic challenges. When an organism perceives one of those challenges as a potentially harmful threat, a suit of physiological and behavioural changes allows the animal to cope with it through the so-called "stress response" (Moberg & Mench, 2000). One of the most studied responses to stress is the activation of the hypothalamic–pituitary–adrenal/interrenal (HPA/HPI) axis. As with other vertebrates, the reptilian hypothalamus releases corticotropin-releasing hormone, which in turn stimulates the adenohypophysis to release adrenocorticotropic hormone (ACTH). Circulating ACTH travels to the adrenal tissue and increases the synthesis and secretion of glucocorticoids (GC), which will act on various target organs and tissues (Moore & Jessop, 2003; Tokarz et al., 2012; Wingfield et al., 2001). A key metabolic function of GC is to mobilize and regulate energy reserves and to restore the homeostatic balance (Dallman et al., 1993; Guillette et al., 1995). After brief and not overly injurious stressors, GC release is regulated via negative feedback and the organism tends to fully recover the homeostatic equilibrium. In healthy individuals, an acute stress response results in short-term allocation of energy without disruption of long-term physiological functions (Dallman & Bhatnagar, 2011; Romero, 2004; Sapolsky et al., 2000). However, in the face of intense or long-lasting threats, the prolonged activation of the HPA axis can disrupt the negative feedback of GC. Consequently, homeostatic restoration can be challenging having, ultimately, detrimental and maladaptive effects on different body systems and negative impacts on health (Mormède et al., 2007; Narayan, 2019; Pahuja & Narayan, 2021). Under chronic stress, therefore, the adaptive mechanisms of the acute stress response shift from having protective roles to causing damage and pathological conditions (Dickens & Romero, 2013). The vertebrate neuroendocrine stress axis, nevertheless, presents a certain degree of plasticity with respect to exposure to certain acute or chronic environmental stressors (Johnstone et al., 2012; Narayan, 2017). There are, therefore, significant inter- and intra-individual differences in the stress response which are potentially influenced by factors such as sex, hierarchy, or season, among others (Cockrem, 2013; Dunlap, 1995). Variation among individuals' acclimation to a specific stressor can favour resilience to stress in some species, whereas increasing vulnerability in others (Dantzer et al., 2014; Owen et al., 2014). Hence, the study of the stress axis may help understand how

different stressors affect the organisms' well-being and identify potential environmental challenges and conservation threats (Marion et al., 2020; Ralph & Tilbrook, 2016). The decline of reptilian populations has been linked to habitat loss and degradation, unsustainable trade, invasive species, and global climate change (Gibbons et al., 2000). To date, 20% of reptilian species are threatened with extinction, and another 20% is classified as Data Deficient following the IUCN Red List Categories and Criteria (Böhm et al., 2013). In this regard, endocrine indicators in reptile species can potentially be applied to predict with accuracy and precision how they respond to stressors and thus help solve conservation problems.

The measurement of the HPA/HPI axis activity and the stress response in reptiles, as in most vertebrates, has been traditionally made by the analysis of GC in blood samples. Blood collection, however, cannot always be performed or may not be the most informative method (Kersey & Dehnhard, 2014; Zemanova, 2020). As demonstrated, the measurement of GC in blood, either at baseline or at stress induced levels, does not reflect chronic stress in a consistent manner (Dickens & Romero, 2013). Repeated blood sampling or the administration of hormone implants or injections, procedures that could better inform about the stress response (Sheriff et al., 2011), are not practical for many species due to conservation or ethical concerns. New approaches wherein GC can be measured are available for the scientific community (Mormède et al., 2007). Seeking to improve such alternative sample types aims at offering advantages over the use of plasma. For instance, preventing the stress triggered by capture, avoiding the influence of the circadian cycle, integrating the measurement of the HPA axis activity over longer timeframes (hours to days, weeks or months), and improving the techniques for endangered species or those that are difficult to capture or handle.

A careful validation for each species and sample of interest is essential before drawing biologically meaningful conclusions, especially when using unconventional techniques (Buchanan & Goldsmith, 2004; Palme et al., 2013). Methodological and analytical validations are performed to assess whether certain conditions, manipulations or other components present in the sample do not significantly influence the validity of the assessments. Therefore, first and foremost, characteristics related to the collection procedures and the analytical methods need to be established on an evidence-based approach. Afterwards, biological, or physiological validation techniques are performed to demonstrate that hormone fluctuations in the specified sample reflect an event of interest. With the commonly called biological validation, GC levels from a control group are usually compared with those in which animals are subjected to a known stressor (such as handling, confinement or transport). Pharmacological experiments performed by using an ACTH challenge or dexamethasone suppression tests are very informative and widely applied as physiological validation (Cook, 2012; Sheriff et al., 2011). Yet, conducting a physiological validation may be excessively invasive in some conditions (Behringer & Deschner, 2017). Alternatively, a biological validation can be more easily achieved by assessing the GC levels before and after exposure to a stressor.

These nonconventional and usually non-invasive sample types (i.e. saliva, faeces, hair, feathers) have been reviewed for all vertebrate classes (Gormally & Romero, 2020; Kersey & Dehnhard, 2014; Sadoul & Geffroy, 2019; Sheriff et al., 2011). However, the reptile perspective has always been limited to a few lines of evidence. Therefore, this brief review attempts to summarize the available biological tissues for measuring GC or their metabolites in reptiles: blood, faeces and integumentary structures (scutes, shed skin and claws) (Table 1). The aim is to inform readers about the application of these relatively novel techniques, to focus attention to where further research is needed, and encourage researchers to use new tools that may help broaden current understanding about reptiles' endocrine system.

Blood

Hormone measurements in reptiles, as in most other vertebrate classes, started with the analysis of GC in blood (Bradshaw & Fontaine-Bertrand, 1970). The widespread knowledge on reptile endocrinology has undoubtedly been accomplished through blood hormone quantifications, the "gold standard" method that still stands out (i.e., Durso et al., 2020).

The circulating measurement of corticosterone (CORT), the main GC in reptiles, offers an instantaneous snapshot view of the HPA axis activity at the time the sample is taken (Sheriff et al., 2011; Van Waeyenberge et al., 2018). This technique has long been an excellent approach to assess reptile responses to different stressors. Of foremost importance for blood collection is the dial and seasonal variation of GC levels and the capture-induced hormone release that may confound the results (Baker et al., 2013; Romero & Reed, 2005). Circadian rhythmicity in GC release has been related to the activity phase of the species, with a morning and an early-night hormonal peak in diurnal and nocturnal animals, respectively (Dickmeis, 2009; Martínez Silvestre, 2014). As demonstrated by the biphasic rhythm in the American alligator (Lance & Lauren, 1984) or the absent diel cycle in tuataras (Anderson et al., 2017; Tyrrell & Cree, 1998), it should be kept in mind that this pattern is not universally consistent. Accordingly, to avoid the variability caused by the circadian cycle, replicate blood samples should be collected at the same sampling timepoint, especially when values need to be related across treatments or in a long-term study. Furthermore, CORT release in reptiles can dramatically change across seasons (Guillette et al., 1995; Neuman-Lee et al., 2020; Romero, 2002; Zena et al., 2020). Hence, potential seasonal variations in the design of endocrine-based studies in this vertebrate group should be always accounted for.

Blood is probably the most informative sample about present GC levels. Nevertheless, to quantify blood CORT levels while avoiding the response elicited by the stress of capture and handling, samples should be obtained within a short timeframe after the animal is captured (Romero, 2002; Wingfield et al., 2001). The delay of hormone secretion after the onset of a stressor has been studied in detail in several reptile species and good reviews are available (Cockrem, 2013; Romero, 2002; Romero & Reed, 2005). The 2–3 min window seems appropriate for most of the species studied so far. However, some species (i.e. rattlesnakes *Crotalus oreganus* or rock iguanas *Cyclura cychlura*) the time period in which to acquire baseline

Table 1 Published studies measuring corticosterone and its metabolites in less- or non-invasive matrixes in different reptile species

Reference	Matrix	Species	Effect evaluated on CORT levels or validation performed
Rittenhouse et al. (2005)	Faeces	*Terrapene carolina triunguis*	Effect of radiotransmitter attachment
Case et al. (2005)	Faeces	*Terrapene carolina carolina*	Effect of environmental enrichment
Suárez-Domínguez et al. (2011)	Faeces	*Ctenosaura acanthura*	Effect of habitat perturbation
Kalliokoski et al. (2012)	Faeces	*Iguana iguana*	Effect of housing and handling
Berkvens et al. (2013)	Faeces	*Lamprophis fuliginosus & Sistrurus catenatus catenatus*	Biological validation
Ganswindt et al. (2014)	Faeces	*Crocodylus niloticus*	Methodological and physiological validation
Halliday et al. (2015)	Faeces	*Thamnophis sirtalis*	Physiological validation; Effect of habitat
Umapathy et al. (2015)	Faeces	*Vijayachelys silvatica & Indotestudo travancorica*	Effect of breeding season
Scheun et al. (2018)	Faeces	*Smaug giganteus*	Methodological and physiological validation
Borgmans et al. (2018)	Faeces	*Anolis carolinensis*	Effect of environmental enrichment
Hudson et al. (2019)	Faeces	*Anolis carolinensis*	Effect of social encounters
Borgmans et al. (2019)	Faeces	*Anolis carolinensis*	Effect of cage size
Kechnebbou et al. (2019)	Faeces	*Uromastyx acanthinura*	Effect of habitat perturbation
Megía-Palma et al. (2020)	Faeces	*Gallotia galloti*	Effect of environmental variability
Augustine et al. (2020)	Faeces	*Crocodylus rhombifer*	Effect of season, sex and reproductive status
Borgmans et al. (2021)	Faeces	*Anolis carolinensis*	Effect of long-term captivity
Martín et al. (2021)	Faeces	*Trogonophis wiegmanni*	Effect of contamination
Kummrow et al. (2021)	Faeces	*Chamaeleo calyptratus*	Effect of reproductive status
Scheun et al. (2021)	Faeces	*Smaug giganteus*	Effect of elevated temperature
Megía-Palma et al. (2022)	Faeces	*Psammodromus algirus*	Effect of temperature and proximity to roads
Martín et al. (2023)	Faeces	*Trogonophis wiegmanni*	Physiological validation
Racine et al. (2022)	Faeces	*Pituophis species*	Effect of indigestible materials in faecal samples
Augustine et al. (2022)	Faeces	Montivipera wagneri	Effect of habitat cleaning
Hartzheim et al. (2023)	Faeces	*Geochelone elegans & Pyxis arachnoides brygooi*	Effect of species
Scheun et al. (2018)	Urine	*Smaug giganteus*	Methodological and physiological validation
Berkvens et al. (2013)	Skin	*Lamprophis fuliginosus & Sistrurus catenatus catenatus*	Biological and physiological validations
Carbajal, Fernández-Bellon, et al. (2014)	Skin	Multiple species	Methodological validation
Carbajal et al. (2018)	Skin	*Varanus komodoensis*	Effect of sex, age, body region and season
Dillon et al. (2021)	Skin	*Salvator merianae & Thamnophis rufipunctatus*	Methodological validation
Zena et al. (2022)	Skin	*Salvator merianae*	Biological validation; Effect of season
Hamilton et al. (2018)	Tail scutes	*Alligator mississippiensis*	Methodological and biological validation
Finger et al. (2019)	Tail scutes	*Alligator mississippiensis*	Effect of selenium exposure
Baxter-Gilbert et al. (2014)	Claws	*Chrysemys picta marginata*	Effect of roadways
Matas et al. (2016)	Claws	*Chamaeleo chamaeleon*	Methodological validation

CORT, Corticosterone; FGM, Faecal Glucocorticoid Metabolites.

CORT levels may be shorter (Tylan et al., 2020). Conversely, evidence strongly suggests that other species present a longer delay, where increases of CORT may not be detectable for up to 10–15 min after the initiation of the disturbance (Neuman-Lee & French, 2017; Romero & Reed, 2005; Tylan et al., 2020).

From healthy reptiles, around 10% of the total blood volume can be safely collected (Sykes & Klaphake, 2015). The body site of blood collection is family or even species-specific, and many venepuncture sites have been described. While the caudal tail vein is the preferred sampling location, other options include the orbital sinus, the jugular vein or cardiac puncture (de la Navarre, 2006; Durso et al., 2020; Sykes & Klaphake, 2015; Tylan et al., 2020). In snakes, the ventral coccygeal vein or performing a cardiocentesis are recurrent techniques, although cardiocentesis can have relevant health risks (i.e., heart contusion or death). The ventral coccygeal vein or the ventral abdominal vein are the preferred sampling sites in lizard species. In chelonians, the venepuncture is usually performed in the jugular vein, dorsal coccygeal vein, or the

supcarapacial sinus, while in small crocodiles, samples are collected from the ventral coccygeal vein, or the supraventral vein in bigger specimens.

As introduced before, the very long history of blood-borne GC research has made this tissue the sample of choice from which to measure hormone levels. Consequently, a wide range of analytical methods, such as EIA, RIA or HPLC, have been successfully validated and applied for different research purposes. Blood collection allows the measurement of a great variety of other physiological parameters; thus, several variables can be simultaneously assessed in a single blood sample (i.e. Durso et al., 2020; Neuman-Lee et al., 2020). To keep the hormone stability, samples should be kept on ice until the collection protocol has finished. Once in the laboratory, they can be centrifuged to obtain serum or plasma, wherein GC are highly stable over time when stored at −20°C. Despite the advantages, blood sampling can be sometimes challenging, and trained personnel is usually required. In addition, blood drawing involves a substantial degree of invasiveness that may not always be possible or desirable. Reptile species present a lymphatics system that commonly runs very close to blood vessels; thus, haemodilution can potentially influence hormone levels (Bonnet et al., 2016). As above mentioned, cardiocentesis in snakes can cause collagen fibrosis and epicardium thickening (Isaza et al., 2004). In small lizards, although the ventral abdominal vein is suggested for blood sampling, risks of vessel and viscera lacerations should be considered (Redrobe & MacDonald, 1999; Sykes & Klaphake, 2015). Repeated blood sampling, a practice useful to track the endocrine activity, is generally not recommended, especially in small individuals where the process itself can damage the vascular beds (Kersey & Dehnhard, 2014). Accordingly, if one wishes to evaluate prolonged stress or monitor the HPA axis over longer time periods in reptiles, blood sampling will probably not be as successful as the samples reviewed below.

Faeces

The preferred non-invasive alternative to blood sampling in reptiles is measuring GC metabolite levels in excreta. Nevertheless, from the first analysis of faecal glucocorticoid metabolites (FGM) in reptiles (Case et al., 2005; Rittenhouse et al., 2005) to date, only few studies have been published using this technique. As revealed by Palme (2019), 12 studies had performed GC measurements on reptilian excreta compared to 274 hormonal studies on mammalian faeces. By the time of the writing of this review, a total of 24 studies using reptiles' faeces for hormone measurement had been published (Table 1). Among these publications, only one author has ventured into the measurement of GC metabolites in urine (Scheun et al., 2018).

Glucocorticoids are mainly metabolized in the liver and excreted via urine or bile (Palme, 2005). In the intestinal tract, GCs excreted via bile may be further metabolized by bacterial enzymes and/or partially reabsorbed (enterohepatic circulation) before being eliminated from the body through faeces and/or urine (Palme, 2019). In reptilian species, products of the digestive and excretory tracts end into the cloaca; thus, urine and faeces are excreted simultaneously although not mixed (O'Rourke & Lertpiriyapong, 2015; Singer, 2003). Commonly, the entire excreted sample is homogenized and used for the hormone quantification. However, it is also possible to remove the urine fraction before analysis of the faecal portion (Kummrow et al., 2010; Myburgh et al., 2012; Scheun et al., 2018). The measurement of FGM provides, therefore, an integrated measure of hormone levels over a period longer than the one offered by the measurement in blood (Goymann, 2005). The time-lag between the onset of the stressor and hormone excretion is highly species-specific and strongly related to the gastrointestinal passage time (Palme et al., 1996; Taylor, 1971). As observed previously, defecation rates in reptiles are not constant and within and between-individual differences can be easily observed (Berkvens et al., 2013; Ganswindt et al., 2014; Kalliokoski et al., 2012; Kummrow et al., 2010). The environmental conditions, the temperature, as well as diet variation across seasons should always be considered given that they can influence the gut passage time (Atkins et al., 2002; Zari, 1993). In reptiles, therefore, the delay between the disturbance and the occurrence of FGM in the excreta is comparatively much more variable than in mammals. Prior studies such as the one performed by Miller et al. (2013) to evaluate the gut passage time of the target species are advisable, as it will provide key information for proper data interpretation.

Evidence in other vertebrates suggests that FGM are influenced by the time between defecation and preservation (reviewed in Sheriff et al., 2011). Accordingly, once samples are collected, they should be stored frozen or processed in the laboratory as soon as possible to avoid bacterial degradation (Palme, 2019). In reptiles, the only existing work that has tested the stability of FGM revealed that faecal levels in crocodiles stayed stable for up to 72 h at ambient temperatures after being excreted (Ganswindt et al., 2014). An additional critical point is the hormone extraction protocol, where validation of the methods is highly recommended (Palme, 2005; Sheriff et al., 2011). Although not mandatory, first step usually involves sample dehydration before its manual or mechanical pulverization. Then, a settled amount of sample is weighted for hormone extraction (ranging from 0.05 to 0.2 g in most reptile studies). Understanding of whether sample mass can influence the final output as well as defining the suitable weight is critical for accurate hormone interpretations (Hayward et al., 2010; Tempel & Gutiérrez, 2004). In this regard, researchers should be aware that, to ensure sufficient faecal material for extraction, samples will probably need to be pooled within individuals. This is especially true for small lizards and some snake species, which can eventually generate meaningless data (Berkvens et al., 2013; Kalliokoski et al., 2012). Another common concern when evaluating FGM is the potential variability caused by indigestible materials that are excreted in faeces, such as hair, bones, and teeth, and this is particularly relevant for snakes. As demonstrated previously in several *Pituophis* species, purifying the faecal sample by removing the indigestible materials can improve up to a 95% the overall yield of CORT levels detected (Racine et al., 2022).

Most published studies preferred extraction of dried faeces with a methanol-based technique at 80–90% rather than using ethanol. Samples are then vortexed for about 15–30 min,

centrifuged, and the supernatant recovered for the measurement of hormone levels. Researchers have favoured the use of EIA as analytical technique for FGM in reptiles against RIA, probably because EIAs do not use radioactive substances neither qualified personnel to be performed (Sheriff et al., 2011). Physiological validation by ACTH injection (Ganswindt et al., 2014; Scheun et al., 2018) or corticosterone administration (Martín et al., 2023) has been successfully performed on several occasions, demonstrating that FGM increases reflect the HPI axis activity, similarly to hormone concentrations in urine (Scheun et al., 2018). Failing to detect a relationship between ACTH administration and FGM levels has been mainly attributed to the irregular reptile defecation pattern (Berkvens et al., 2013). However, the proportion of CORT metabolites in faeces of common gartersnakes has been successfully related to their abundance in bloodstream, further increasing the applicability of this non-invasive technique (Halliday et al., 2015).

Faecal sampling offers the obvious advantage that its collection is non-invasive, usually based on a simple sampling protocol; thus, no qualified personnel is needed. Unlike blood, the collection of faeces does not necessarily influence the HPA axis, allowing the measurement of baseline hormone levels. Moreover, repeated faecal sampling is usually possible enabling long-term hormone monitoring. Overall, FGM have been proposed to be good indicators of the reptile stress response to handling and restraint (Kalliokoski et al., 2012). Moreover, levels of FGM have been successfully correlated to various social and environmental factors (Ganswindt et al., 2014; Hudson et al., 2019; Kalliokoski et al., 2012; Kechnebbou et al., 2019; Megía-Palma et al., 2022; Suárez-Domínguez et al., 2011), and to fluctuate seasonally along with the breeding season (Umapathy et al., 2015). Importantly, infrequent defecation in this vertebrate group hinders the use of FGM as a daytime physiological indicator. Therefore, researchers should avoid the use of faecal hormone levels to evaluate daily processes and instead exploit its potential to elucidate cause-and-effect relationships over longer time periods.

Keratinized tissues

Glucocorticoid hormones have also been extensively quantified in keratinous tissues as a measure of long-term HPA axis activity. These samples include hair (Cirimele et al., 2000; Heimbürge et al., 2019; Tallo-Parra et al., 2015), feathers (Bortolotti et al., 2008; Carbajal, Tallo-Parra, et al., 2014; Romero & Fairhurst, 2016), baleen (Hunt et al., 2017) or less frequently nails and claws (Fusi et al., 2018; Warnock et al., 2010). Similar measures in reptiles have been attempted in scutes and in shed skin, the homologous tissue to mammals' hair and birds' feathers, along with the analysis of hormone levels in claws.

The process of ecdysis, or skin shedding, is a dynamic cycle that occurs periodically throughout the whole life of the animal (Cooper, 2006). While snakes usually shed in one piece, lizards renew their skin in patches, and turtles and crocodiles shed individual scutes (Girling, 2013). Once the shedding cycle has finished and the old skin has been shed, the new cycle starts. The hypothesis is that circulating hormones are deposited in the new epidermal layer while it is being formed, and potentially, during the time it is in place until the upcoming skin shedding process begins (Berkvens et al., 2013; Zena et al., 2022). In light of this, levels of CORT have been measured in crocodile tail scutes (Hamilton et al., 2018) and in the shed skin of various snake and lizard species (Berkvens et al., 2013; Carbajal et al., 2018; Carbajal, Fernández-Bellon, et al., 2014; Zena et al., 2022). Increases of CORT levels in these keratinous tissues are thought to reflect prolonged or repeated stress exposures, as described in American alligator (*Alligator mississippiensis*) subjected to long-term environmental contamination (Finger et al., 2019). However, contrary to expectations, CORT levels in tail scutes of the same species increased in response to a 2-h handling stress, thus a single and relatively short-term stressor (Hamilton et al., 2018). The authors discussed that hormone levels in scutes could be in relation to the sample depth and its adjacency to the vascularized tissue. A potential variation between scutes was also considered, evidencing the importance of both methodological and validation studies. Accordingly, these studies suggest that CORT concentrations in scutes can be influenced by a stimulus as short of 2 h long to a long-term exposure of at least 7 weeks of duration.

The only validation study performed so far, did not detect higher shed skin GC levels in daily stressed snakes compared to control animals (Berkvens et al., 2013).

The same authors also performed an ACTH challenge where the injection of ACTH should provoke the secretion of circulating GC and increase CORT levels in shed skin. However, levels in shed skin of African house snakes did not increase as expected. Despite that, the authors revealed a positive association in CORT levels between shed skin and faeces providing, albeit modest, grounds for hope for the use of this alternative non-invasive sample. Further proof that this tool shows promise was given by the biological variation identified in shed skin CORT levels of Komodo dragons (Carbajal et al., 2018). Hormonal patterns in Komodo' shed skin were related to sex, age and season, revealing that there are potential variables that should be considered to overcome confounding influences, as also supported by Zena et al. (2022).

Following the same hypothesis above mentioned, relative to GC accumulation in keratinized tissues, claws are thought to incorporate CORT while the tissue is growing through diffusion from capillaries (Baxter-Gilbert et al., 2014). To date, CORT from claws has been successfully measured in turtle (Baxter-Gilbert et al., 2014) and chameleon (Matas et al., 2016). No relation was found between claw CORT levels and presence near major roads, but levels were higher in males than in females (Baxter-Gilbert et al., 2014). Like tail scutes collected in crocodiles, claw clipping is applied for individual identification; thus, their sampling offers at least dual application. Despite the great potential this tool may have, methodological, biological, and physiological validations are still lacking.

Collection methods of keratinous samples need further improvement. As demonstrated so far, body region and tissue depth can influence hormone levels in some species, which can challenge the understanding of endocrine data (Berkvens

et al., 2013; Hamilton et al., 2018). Extraction techniques are generally based on protocols previously described for other keratinous samples. In brief, samples are externally cleaned with distilled or ultra-pure water followed by a bath with diluted methanol. Once dry, they are manually or mechanically grinded. For hormone extraction, samples are incubated in 80–100% methanol from 18 to 24 h in an orbital shaker. The accurate sample mass has not been examined yet, but evidence in chameleons' claws suggests that small amounts (0.07 mg) can still provide relevant data (Matas et al., 2016). All published studies to date agree that EIA is probably the most appropriate technique by demonstrating the reliability of the analytical method for each steroid and species of interest.

Keratinous tissues in reptiles show promise as an alternative way to measure hormone concentrations integrated over time. They can be collected in a relatively non-invasive way, following a comparatively simple protocol. Samples are easy to store in plastic or paper envelops at room temperature, a great advantage under field conditions. Other hormone measurements can be made simultaneously in the same sample, and as previously described, samples can be used as indicators of toxicant exposure (Finger et al., 2019). Nevertheless, more work is required to further validate these techniques. Performing biological and/or physiological validation is imperative to understand whether short and long-term changes in GC are reflected by the measured hormone in these samples afterwards. Studies focused on the period of time represented by the keratinized sample are equally important as those concerned over issues related to the collection and preparation techniques.

Other non-lethal sample media

Other potential means of assessing the HPI axis activity in reptiles throughout GC measurement might eventually be possible, although still unexplored. For example, saliva has been extensively used in mammals as an alternative sample to measure GC levels (Cook, 2012; Sheriff et al., 2011). In reptilian species, buccal swabbing has proved to provide reliable genetic (Beebee, 2008; Mucci et al., 2014) and toxicological (Mingo et al., 2019) data. Besides, our research group has detected salivary sex hormones in an on-going study on Komodo dragons (unpublished data). Similarly, 17β-estradiol and testosterone accumulated in the eggshell of loggerhead sea turtles (*Caretta caretta*) can be extracted and measured (Kobayashi et al., 2015). These observations suggest that both saliva and eggshells of reptiles could offer a non-invasive measure of the HPI axis activity. Recent evidence indicates that CORT levels can also be measured in cloacal swabs and salt gland secretions of marine turtles, and probably, levels of CORT in these samples are in proportion to their abundance in the bloodstream (Carbajal et al., 2019). Other potential non-invasive GC measures may be available in reptiles, but they still need to be considered and examined. Importantly, these techniques should be fully validated to demonstrate that the measure can provide biologically meaningful data and settle accurate interpretations.

Conclusion

The present review demonstrates that several unconventional and relatively non-invasive measures of GC are available to study the reptiles' endocrinology, although the potential of these samples is underexploited. This is not surprising since much of the hormone-related work has focused on fish, mammals and birds, and few research has centred attention on reptiles' and amphibians' endocrinology. With more than one-fifth of reptilian species threatened with extinction, revealing whether and how individuals cope with a changing environment is critically important. The endocrine techniques presented here may contribute to deepen our understanding of the physiological responses to anthropogenic disturbances, and therefore allow the advancement of reptile conservation physiology.

Author contributions

Annaïs Carbajal and Manel López-Béjar conceived the ideas and described parts of the review; Annaïs Carbajal and Sergi Olvera-Maneu performed the literature search; Annaïs Carbajal and Paula Serres-Corral analysed the published literature; Annaïs Carbajal led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Conflict of interest

All authors confirm that there is not any conflict of interest.

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