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Short communication

A method to determine integrated steroid levels in wildlife claws

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ABSTRACT

Glucocorticoids act throughout life to regulate numerous physiological and behavioral processes. Their levels are therefore highly labile, reacting to varying conditions and stressors. Hence, measuring glucocorticoids (and other steroids) in wildlife is challenging, and devising methods that are unaffected by the stress of capture and handling should be explored. Here we use the tip of free-ranging chameleons' claws that were cut to allow individual identification, and report a steroids extraction and quantification method. Claw steroids present an integrated level representing the period of claw growth. We found that we could measure corticosterone in small amounts of chameleon claw matrix using commercial EIA kits. Using this method, we learned that in wild male chameleons, claw corticosterone levels were associated with body size. We suggest that claw-testing can potentially provide an ideal matrix for wildlife biomonitoring.

1. Introduction

Using hair and feathers to quantify steroid levels has opened a window into understanding long-term individual variability in wildlife (Koren et al., 2002). Hair and feather collection do not necessitate capture and handling and tissue from culled specimens can be used (Bryan et al., 2015). Here, we evaluate whether reptilian claws can potentially provide a source of integrated steroid levels in wildlife. Claws can be easily collected from captive wildlife in a relatively non-invasive manner. Similar to hair and feather, samples are safe to handle, and storage is at room temperature; ideal for field conditions.

In mammals, claws and nails develop by keratinocytes cell divisions in the proximal germinal regions (Baran, 1981; De Berker et al., 2007). Nail growth is attributed primarily to the nail matrix and to a lesser extent to the nail bed (De Berker et al., 1996). However, it appears that this growth pattern is not a general phenomenon in vertebrates. The development of claws in several reptiles was demonstrated to result from the modification of terminal scales (Alibardi, 2010). In an elegant study, Alibardi showed that reptilian cell proliferation occurs primarily in the dorsal side of the end of the digit, giving rise to the curved shape of the claw (Alibardi, 2010). Although the study included only several reptile species, it may be possible to generalize to all Reptilia, since it showed that reptilian claw structure of the examined representative reptiles is histologically identical. Furthermore, studies suggest that avian claws develop in a similar process (Krejsa, 1979).

Diverse factors influence nail growth-rate, including various physiological and pathological conditions (reviewed in (Zaiac and Walker, 2013)). On average, human fingernails grow at a rate of 3 mm per month, while human toenails grow 1 mm per month. Much less is known regarding claw growth rate. Several studies have documented claw growth rate in birds (Hahn et al., 2014). However, we could not find data for claw growth kinetics in other classes.

As nails and claws grow, materials can be transferred from the blood (Palmeri et al., 2000). These, along with the availability of these matrices make them attractive candidates for biomonitoring. Indeed, human nails have been utilized for decades in forensic examinations to detect exposure to poisons (Barbosa et al., 2005; Button et al., 2009; Mehra and Juneja, 2005; Suzuki et al., 1989). Recently, the possible usage of nails for the detection of exposure to endocrine-disrupting chemicals gained empirical support (Li et al., 2013). Several studies also used nails for endogenous steroid quantitation. DHEA (dehydroepiandrosterone) extracted from nails of infants who experienced stress during pregnancy was higher compared to infants from non-stressed mothers (Tegethoff et al., 2011). Additionally, cortisol-to-DHEA ratio was found in correlation to perceived exam stress in students (Warnock et al., 2010). Measurement of testosterone and its derivatives for the detection of doping was also reported (Brown and Perrett, 2011). However, this study concluded that detection of these substances from nails is not sensitive enough.

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Recent studies suggest that claws may offer a potential matrix for biomonitoring wildlife. Cortisol in newborn dog claws was shown to associate with hair cortisol (Veronesi et al., 2015). Turtle claw corticosterone was higher in males than in females but no association was found between claw corticosterone and presence near major roads (Baxter-Gilbert et al., 2014). Clearly, claw steroid quantitation requires further validation and testing in additional species. Here, as an example of the utility of this method, we use commercial ELISA kits to measure corticosterone in common chameleon (*Chamaeleo chamaeleon*) claws.

2. Materials and methods

2.1. Study animals, study site and data collection

The common chameleon occupies park forests and plantations in the Mediterranean region of Israel and southern Spain. We conducted this study along the Maharal creek on the Mediterranean coast, at the foothills of Mt. Carmel, Israel (32°38′ N, 34°58′ E), where chameleons are active mostly during the warm months (May–November). The mean maximum and minimum daily temperatures at this time are 30 and 21 °C, respectively, and relative humidity averages 70%. Mean annual rainfall at this location is 550 mm, accumulating November–March. Fieldwork was carried out May–December 2008–2012.

Chameleons were collected during the breeding period (August–October) from the vegetation during the night, using a spotlight. A total of 44 sexually mature adult males were used in this study. To minimize stress, chameleons were kept for up to 24 h in separate 35 × 20 cm terraria, placed in a shady area inside a screen cage to prevent predation. Keeping animals outdoors maintained natural conditions (e.g., air temperature and humidity). We did not provide food or water because the animals were held during the nighttime, when they are not active, and released a few hours after sunrise.

All the individuals were weighed and sexed according to the presence or absence of male hemipenial organs. We individually marked all captures prior to release by clipping the tip of 1–3 claws using a fingernail cutter. This procedure lasted a few seconds, and hand-held animals showed no resistance. The clipped claws regrew a rougher tip, which served to identify recaptures but did not affect the animals' ability to climb branches (Cuadrado, 2000).

2.2. Steroid extraction

Similar to claws, hair is a keratin-based matrix. We extracted steroids from claws using our published protocol for hair-testing (Koren and Geffen, 2009a,b; Koren et al., 2006, 2008, 2002, 2012), with several modifications. Claw clippings were weighed to the nearest 0.01 mg in a safe-lock polypropylene tube (Sarstedt, Germany). Methanol (UPLC grade, Sigma, Israel) was added to the samples. Then, samples were ground in mixer mill MM 400 (Retsch, Germany) for 2 min at 25 Hz. Next, samples were sonicated for 30 min and incubated overnight at 50 °C with gentle shaking. Next day, methanol was transferred into a glass tube (Corning Inc., USA) and evaporated under a stream of nitrogen. Samples were reconstituted in assay buffer provided in the commercial ELISA kit.

2.3. Quantitation of steroids

Corticosterone was quantitated in claw extracts using commercial enzyme-linked immunosorbent assays (ELISA) according to manufacturer's recommendations (Cayman Chemical Company item no. 500655; Ann Arbor, USA). Serial dilutions of a pool of claw samples showed parallelism (univariate analysis of variance; $p = 0.55$), and provided a linearity range between 120 and 1000 pg/mL, equivalent to 0.5–7.5 mg claws. The lowest concentration we detected by the assay was 6.6 pg/mL, corresponding to 0.07 mg claws. According to the manufacturers, the corticosterone antibody cross-reacts with 11-dehydrocorticosterone (11%), 11-deoxycorticosterone (7%) and other steroids (⩽0.31%). Intra-assay variability was determined to be 2.4% by quantifying multiple samples of the pool on the same ELISA plate ($n = 4$). Inter-assay variability was calculated to be 14.9%, by running multiple samples of the pool on different days ($n = 4$). Extraction efficiency per se cannot be obtained for claws, since it is not possible to spike into the matrix itself. However, we examined recovery by the addition of a known amount of corticosterone to the claw sample and obtained 88% efficiency. The presence of corticosterone in chameleon claws was further validated utilizing a second antibody, by the ENZO corticosterone ELISA kit (Enzo Life Sciences item no. ADI-900-097; NY, USA) following the manufacturer's protocol. ENZO reports that the corticosterone antibody cross-reacts with deoxycorticosterone (28.6%), progesterone (1.7%) and other steroids (⩽0.28%).

2.4. Statistics

In order to account for variability across plates, we standardized steroid concentrations within the plate. Then, we used the residual from the association between standardized steroid concentrations and claw weight (in mg).

3. Results and discussion

Here we report for the first time quantitation of steroids from chameleon claws. We were able to measure corticosterone in claw extracts that originated from minute samples of claw clippings, weighing 0.07–1.38 mg (mean ± SD = 0.52 ± 0.3 mg). In order to biologically validate our method, we related male corticosterone levels to body weight. Almost 10% of the variation in corticosterone levels was explained by male body weight ($R^2 = 0.0997$; $n = 45$; $p = 0.03$; Fig. 1), so that heavier males had higher claw corticosterone levels.

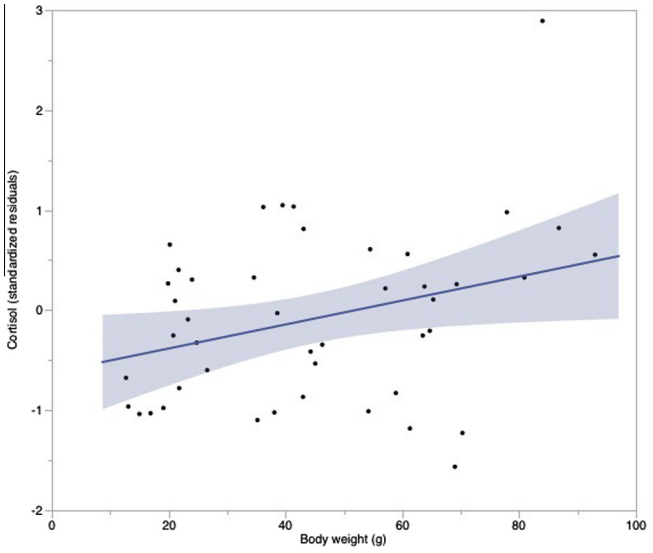

Fig. 1. The association between male chameleon claw corticosterone and body weight. Claw corticosterone levels are standardized residuals corrected for claw weight. $R^2 = 0.0997$; $n = 45$; $p = 0.03$.

Common chameleon steroid levels have not been previously reported, yet studies on several other species supported the positive association that we found between body size and corticosterone. In several lizard species (e.g., *Anolis carolinensis*; *Urosaurus ornatus*), as well as other species, body size and weight are related to contest outcome and dominance (Drickamer et al., 1973; Fournier and Festa-Bianchet, 1995; Jenssen et al., 2005; Zucker and Murray, 1996). Even in humans, a positive correlation was demonstrated between urinary corticosterone metabolites and body size. Common chameleon males use alternative reproductive tactics related to male body size and weight. We recently found that larger and heavier males were dominant while smaller and lighter males were subordinate (TKR unpublished data). These different reproductive tactics among chameleon males were also found to characterize various behavioral patterns such as perch height preference, and body inflation during social encounters (TKR unpublished data). Since social dominance is related to glucocorticoids in many vertebrates (Creel, 2001; Sapolsky, 1982), an association between glucocorticoids and body size or weight can be expected (Tokarz, 1987).

Measurement of steroid levels in claw samples provides a relatively non-invasive alternative for wildlife, causing minimal stress during sample acquisition. Furthermore, while blood steroid concentrations reflect a temporary value, claws provide a long-term record of steroid hormone concentrations integrated over the period of claw growth. Nevertheless, it should be taken into account that the amount of claws that can be sampled from each individual is very small in chameleons. Moreover, since claws are very important for climbing and prey capture, only a small fraction of the claw should be collected. Although the amount of claw tissue for most of the samples in our study was very low, corticosterone was successfully quantified with our extraction and measurement methods. For larger lizards, larger samples may be possible to obtain, allowing quantification of multiple steroids. The application of this method for wildlife research will allow monitoring long-term trends and steroid profiles in reptiles in a relatively non-invasive manner. Steroids quantitation in wildlife may serve to support environmental decision-making regarding wildlife population management. These include hunting-related policies (Bryan et al., 2015), human disturbances such as vehicle noise (Ciuti et al., 2012) and population management (Haber, 1996).

4. Ethics statement

The common chameleon is a protected animal under Israeli law. Collecting them from the wild for our behavioral experiments required a permit from the Israel Nature and Parks Authority, which we were granted annually (permit no. 37394/2010, 38014/2011, 38579/2012). This study complies with all Israel regulations on ethical treatment of wild animals in scientific research.

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