Scales of our lives: sex identification of Temminck's pangolin (*Smutsia temminckii*) using scales retrieved out of the illegal wildlife trade

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Declarations of interest: none

Abstract

Pangolins are the most trafficked wild mammals, with their scales in high demand. Scales are often the only part of the animal confiscated from the trade, but they represent accessible material for forensic investigations, including for sexing. This study aimed to develop a sexing tool for Temminck's pangolin, using scales for hormone quantification. Scales from males and females were liquidised using keratinase and the resulting suspension analysed for progestagen and androgen metabolite (scPM and scAM) concentrations. Scale PM and scAM concentrations were compared between sexes, while overall median values for scPM and scAM, as well as a ratio of scPM to scAM (P/A) were used as boundary values for sex identification. Neither scPM nor scAM concentrations were significantly different between the sexes and concentrations of a juvenile and sub-adult male overlapped with females, possibly indicating later sexual maturity in males. Boundary values for scAM concentrations and the P/A ratio predicted sex with 100% accuracy for females and 78% for males, while the accuracies for the scPM boundary value were lower. When only adult individuals are considered, scAM and P/A ratio boundaries are 100% accurate for both sexes. Therefore, scale hormone ratios show promise as a sex identification tool for Temminck's pangolin, particularly applicable in forensic investigations on the pangolin trade.

Keywords: ground pangolin, *Smutsia temminckii*, pangolin scales, sex identification, progestagens, androgens

1. Introduction

The increasing global wildlife trade causes significant losses to biodiversity and includes around 18% of extant terrestrial vertebrate species, with mammals and their skin derivatives and appendages being the most commonly traded items (Alacs et al., 2010; Roe, 2008; Rosen and Smith, 2010; Scheffers et al., 2019). One mitigation tool is wildlife forensics, which is used to aid with enforcement against wildlife criminals (Ahlers et al., 2017; Gupta, 2018). Genetic analysis forms a major part of wildlife forensics and is used to determine species identity, geographic origin, individual identity, parentage, and sex of traded wildlife (Ahlers et al., 2017; Alacs et al., 2010; Johnson et al., 2014; UNODC, 2016). Sex identification can aid in elucidating demographic differences between sexes, but also in monitoring trade presence of the sexes (Alacs et al., 2010; Tawichasri et al., 2017). Although sex determination is often conducted through genetic analysis, endocrine analysis may serve as an alternative approach, where gonadal hormones are used to identify sex. This approach has been successfully used in various species of reptiles (Gregory and Schmid, 2001; Morris et al., 1996), fish (Mola et al., 2011; Wheeler et al., 2016), anurans (Germano et al., 2009; Graham et al., 2016), and birds (Bautista et al., 2013; Bercovitz et al., 1978). However, this technique is less popular for sexing mammals, with limited examples including wolves (Barja et al., 2008; Velloso et al., 1998), brown bears (*Ursus arctos*) (Cattet et al., 2017), Canada lynx (*Lynx canadensis*) (Terwissen et al., 2014), and domestic cats (*Felis catus*) (Terwissen et al., 2014).

Although hormones and their metabolites are most often quantified in blood and excreta, hair and feathers are becoming increasingly popular matrices (e.g. Carlitz et al., 2015; Cattet et al., 2018; Lattin et al., 2011; Romero and Fairhurst, 2016). Keratinous materials can be stored immediately after collection and hormones and their metabolites appear to be stable and remain detectable even after long storage times, which is an advantage over excreta and blood (Hunt et al., 2014). Moreover, it is suggested that keratins accumulate steroid metabolites during their continuous growth, thereby representing hormone activity over the growth period of the material (Bortolotti et al., 2008; Kersey and Dehnhard, 2014; Palme, 2012; Sheriff et al., 2011). However,

scAM – scale androgen metabolite
scDW – scale dry weight
scPM – scale progestagen metabolite

contrasting studies argue that steroids may diffuse through keratins like hair and are thus more likely to reflect recent and not past events (Colding-Jørgensen et al., 2020; Kalliokoski et al., 2019). Even though more research is needed on the deposition of hormones into keratin-based matrices, it presents an easily accessible sampling material. In most cases, keratin materials are pulverised before hormone extraction (e.g. Baxter-Gilbert et al., 2014; Davenport et al., 2006; Hunt et al., 2014). However, a commercial keratinase enzyme generally used in the feed and fertiliser industry, in detergents and cleaning products, for recycling of keratinous wastes, for cosmetic treatments, and in various biomedical treatments (Brandelli et al., 2010; De Oliveira Martinez et al., 2020; Gupta and Ramnani, 2006; ul Haq and Akram, 2018), has already been used to successfully degrade and extract hormone metabolites from chicken feathers (Alba et al., 2019).

Pangolins (Pholidota, Manidae) are unique among mammals as they are covered in keratin scales, making up almost 20% of their body weight (Liu et al., 2016). The scales are highly sought after in the illegal international pangolin trade for use in various traditional medicine practices and other cultural rituals (Bräutigam et al., 1994; Hua et al., 2015; Soewu and Sodeinde, 2015). This demand has led to drastic population declines of all eight extant pangolin species and the classification of pangolins as the most trafficked wild mammal on earth (Challender et al., 2014; Challender et al., 2020; IUCN, 2020). Despite this, they remain understudied due to their elusive nature (Challender et al., 2014).

Temminck's pangolin (*Smutsia temminckii*) is the only pangolin species found in southern Africa (Heath, 1992; Skinner and Chimimba, 2005). Most of the research on this species has focussed on their ecology (Heath and Coulson, 1997b; Pietersen et al., 2014), diet and feeding behaviour (Pietersen et al., 2016; Richer et al., 1997; Swart et al., 1999), as well as translocation success (Heath and Coulson, 1997a). In contrast, little is known about their general and reproductive physiology. It is rare for confiscations within the illegal wildlife trade to include living pangolin individuals, but rather entire carcasses most often scales; the latter two, however, allow for easy sample collection compared to living individuals. Occasionally, whole pangolin carcasses are seized and morphological sexing is possible, but generally, only scales and parts of carcasses are available and genetic analysis must be employed (du Toit et al., 2020; Luczon et al., 2016; Mwale et al., 2016).

The development of new forensic techniques will aid investigations into the illegal pangolin trade. For example, knowing ratios of males and females present in the trade may elucidate whether the trade is biased towards one sex of this species and in future allow improved law enforcement to protect valuable individuals such as breeding females. As such, the current study was the first to employ an endocrine approach and investigate the suitability of pangolin scales for sex steroid analyses as a sexing method.

2. Material and methods

2.1 Scale collection

Scales were obtained from Temminck's pangolin carcasses, confiscated from the illegal wildlife trade, and stored at a secure facility within South Africa. A total of five females (two adults, one sub-adult, and two juveniles) and nine males (six adults, two sub-adults, and one juvenile) were sampled. Sex was determined morphologically (presence of male or female genitals), while division into age classes was done based on weight. Individuals weighing less than 3.50 kg were classified as juveniles, those weighing between 3.50 and 6.50 kg as sub-adults, and individuals with a weight greater than 6.50 kg were classified as adults (R Jansen, African Pangolin Working Group, pers. comm.). From each carcass, one scale was cut from four body regions: top of the tail base, the middle of the head, and the right and left sides of the body (Fig.1A). From two of the carcasses (one adult female and one adult male), only three scales were collected as the head had been removed. All sampling was approved by the University of Pretoria Animal Ethics Committee (NAS078/2019) and the South African National Biodiversity Institute (SANBI) Research Ethics and Scientific Committee (NZG/RES/P18/06).

2.2 Degradation of scales with keratinase

Scales were submerged in ultrapure water overnight to soften and facilitate easy removal of residual tissues on the scales. The base section (area where the scale was attached to the epidermis of the pangolin) of each scale was then cut (Fig. 1B), with resulting pieces (0.30 - 0.42 g) being used for hormone extraction. If a scale had a mass of less than 0.40 g, the whole scale was utilised (5 out of

54 scales). Scale degradation was based on a protocol first developed by Alba et al. (2019) for chicken feathers and validated for Temminck's pangolin scales by Blecher et al. (2021). In brief, scale samples were washed in 100% isopropanol for 30 seconds, using a toothbrush, to remove surface contaminants. For degradation, each sample was mixed with 15 ml of keratinase solution (1 g enzyme (Cibenza IND900, Novus International, Inc., Missouri) in 30 ml phosphate-buffered saline (pH = 9.00)) and incubated at 45 °C for four days, stirred every 24 hours. Resulting suspensions were stored frozen at -20 °C until hormone analysis.

2.3 Enzyme Immunoassays (EIAs)

Immunoreactive scale progestagen metabolite (scPM) and scale androgen metabolite (scAM) concentrations were measured directly in the digested sample suspensions using a progestagen (Schwarzenberger et al., 1996, antisera raised in rabbits against 5β-pregnane-3α-ol-20-one-2HS:BSA) and androgen (Palme and Möstl, 1994, antisera raised in rabbits against testosterone-3-CMO:BSA) enzyme-immunoassay (EIA), respectively. EIAs were performed following established protocols (Ganswindt et al., 2002), directly on digested scale suspensions. In brief, 50 µl of either standards, quality controls, or digested sample suspension were added in duplicates to pre-coated 96-well microtiter plate wells. Next, 50 µl of biotin-labelled steroid, as well as antibody solution, were added to each well before plates were incubated at 4 °C overnight. The liquid was then discarded and the plate washed four times, after which 150 µl of streptavidin- POD solution was added to every well and the plate incubated with gentle shaking at 4 °C for 45 min. Subsequently, all liquid was again discarded and the plate washed four times before 150 µl of substrate solution was added to every well and incubated with gentle shaking at 4 °C, until the optical density reached ~1.0 for maximal binding. Finally, the enzymatic reaction was terminated by adding 50 µl H_2SO_4 (2 M) to the wells and optical density was determined at 450 nm and 620 nm and results calculated using a best-fit curve.

Both EIAs were biologically validated in Blecher et al. (2021) and their sensitivities were 12 ng/g scale dry weight (scDW) for the progestagen and 1.50 ng/g scDW for the androgen assay. Serial dilutions of scale extracts gave displacement curves that were parallel to respective standard curves (relative variation of the slope of the trend lines was < 1% for the progestagen and < 2 % for the

androgen EIA). Intra-assay coefficient of variations (CV), determined by repeated measurements of high and low value quality controls, were 5.64 % and 6.12 % for the progestagen, and 5.22 % and 6.82 % for the androgen EIA, respectively. Similarly determined inter-assay CVs were 12.33 % and 14.19 % for the progestagen, and 11.27 % and 13.14 % for the androgen EIA, respectively. All assays were performed at the Endocrine Research Laboratory, University of Pretoria.

2.4 Statistical analyses

Differences in scPM and scAM between body regions were previously investigated and found to not differ significantly (Blecher et al., 2021), thus median scPM and scAM concentrations were calculated for each individual. Additionally, a unit-less ratio of scPM to scAM concentration (P/A) was calculated for each individual by division of scPM by scAM concentration, similar to previous studies (Barja et al., 2008; Rolland et al., 2005). Normality was tested using a Shapiro-Wilk normality test and homogeneity of variances was evaluated using Levene's test (R package 'car' (Fox and Weisberg, 2011)). Non-parametric Wilcoxon rank-sum tests were used to compare scPM and scAM concentrations between sexes, as medians more accurately represented the centre of the distribution of the data.

To investigate accuracy of sex prediction using scPM concentration, overall individual median scPM concentrations were calculated for females and males and the median of these two values was used as boundary value. Scales with scPM concentrations above this value were classified as female, while scales with concentrations below this value were classified as male. Similarly, overall individual median scAM concentrations were determined for females and males, and the median of these two concentrations was used as a boundary value. Scales with scAM concentrations above this boundary value were classified as male, while those below were classified as female. For sex prediction with the P/A ratio, the boundary value was calculated by dividing the previously calculated scPM boundary concentration with the scAM boundary concentration, and scales with a ratio values above this were classified as female and below this value as male. Accuracies were calculated as a percentage and all data analysis was conducted using RStudio (R Core Team, 2020), while significance was taken at $p < 0.05$.

3. Results

The scPM concentration was not significantly different (W = 36, p = 0.08, n = 14) between females (median = 441.73 ng/g scDW; range: 348.23 - 481.06 ng/g scDW) and males (median = 316.23 ng/g scDW; range: 270.88 - 512.15 ng/g scDW). Similarly, there was no significant difference (W = 9, p = 0.08, n = 14) in scAM concentration between males (median = 134.73 ng/g scDW; range: 63.16 - 219.67 ng/g scDW) and females (median = 85.56 ng/g scDW; range: 77.04 - 100.99 ng/g scDW).

When using scPM concentration to predict sex (boundary value = 379 ng/g scDW), 80% of females (4/5) and 67% of males (6/9) were correctly assigned (Fig. 2). In contrast, the scAM value (boundary value = 110 ng/g scDW) correctly predicted 78% of males (7/9) and 100% of females (5/5) (Fig. 2). For sex prediction with the P/A ratio (boundary value = 3.44), sex was predicted with 100% accuracy for females (5/5) and 78% for males (7/9). Furthermore, there was a distinction into two groups (one male and one female) when scPM and scAM concentrations are plotted, except for two outlier males that fall into the female group (Fig. 2). These two males consist of one sub-adult, with the highest scPM concentration, and a juvenile male with the lowest scAM concentration of all animals.

We also investigated whether sex prediction accuracies increased if only adults are considered. For scPM concentrations (new boundary value = 388 ng/g scDW) sex prediction was 100% for females (2/2) and 83% for males (5/6), while both scAM concentration (new boundary value = 122 ng/g scDW) and the P/A ratio (new boundary value = 3.18) allowed sex determination to 100% for both adult females (2/2) and males (6/6).

4. Discussion

This is the first study to implement an established method for determining sex hormone concentrations in scales of Temminck's pangolin. Results indicated that scPM and scAM levels differ considerably between the sexes, though a large degree of individual variation was also observed. This is in line with findings from previous studies on other species where similar variation in reproductive steroid concentrations have been shown between individuals and sexes. (e.g. Germano et al., 2012; Guarniero et al., 2017; Oates et al., 2002; Terwissen et al., 2014). Although

sex prediction was more accurate using the scAM boundary compared to the scPM boundary, neither achieved 100% accuracy for both sexes. Similar results have been reported for loggerhead turtles (*Caretta caretta*) (Gross et al., 1995) and dusky gopher frogs (*Lithobates sevosa*) (Graham et al., 2016) when employing endocrine monitoring. As individual hormone metabolite values are variable and often not accurate for sex prediction, the hormone ratio method may be more useful. However, sex prediction accuracies with the P/A ratio were equal to those of the scAM boundary. Some studies have shown that use of a sex steroid ratio is more accurate for sex prediction than the use of single sex steroids (e.g. Graham et al., 2016; Gross et al., 1995; Wasser and Hunt, 2005), while others demonstrated that only single sex steroids concentrations are needed for accurate sexing (e.g. Germano et al., 2012; Germano et al., 2009). Therefore, in Temminck's pangolins, both may be suitable for use.

Individual median hormone values placed within a scatterplot indicated a male and a female cluster. This was also observed in Iberian wolves (*Canis lupus signatus*) (Barja et al., 2008) and great bustards (*Otis tarda*) (Bautista et al., 2013). Moreover, this scatterplot revealed two males that plotted within the female group, which lead to the lower accuracies of males in comparison to females with both the scAM and the P/A ratio boundaries. Such 'outlier' males were similarly found in bell frogs (*Litoria raniformis*) (Germano et al., 2009) and dusky gopher frogs (Graham et al., 2016). As the outliers in the present study consisted of non-adult individuals, these individuals possibly had not reached sexual maturity. Both male and female Temminck's pangolins are thought to reach sexual maturity around the age of two years, however, males often take longer to establish home ranges and potentially only start breeding at the age of six or seven (Pietersen, 2013; Pietersen et al., 2020). Therefore, the rise in androgen levels often accompanying the onset of breeding in male mammals, may only occur once a home range is established and cycling females are encountered. Such a proposed delay in maturation may thus be responsible for the comparatively low scAM concentrations observed in these two males. The other sub-adult male fell within the male group and had possibly already started breeding. This individual may also have been an underweight breeding adult and was incorrectly classified as a sub-adult, as the ages of the pangolins in the present study were determined based on body weight. In addition to low scAM concentrations, these young males

also displayed high scPM concentrations. Some previous studies have shown that administered high doses of progesterone or progestins suppress androgen-dependent sexual behaviours in various mammals, birds and humans (Andersen and Tufik, 2006; Oettel and Mukhopadhyay, 2004; Wagner, 2006; Witt et al., 1994). While young male Temminck's pangolins are in search of their new home range, the high levels of progestagens may be contributing to the delay in sexual maturation proposed here. The two juvenile females in the present study had lower scPM concentrations than the sub-adult and adult females, again possibly indicating that they had not reached sexual maturity and scPM concentrations had not yet increased, as they do in mammalian females upon sexual maturity. While these explanations are speculative, sex steroid analysis in scales may be expanded to investigate the unknown endocrine aspects of sexual maturity in Temminck's pangolin.

Although sexing accuracies were high when the scAM or P/A ratio boundary were used, outliers are possible and should be considered, as with all such techniques. Therefore, we investigated whether accuracies improved if only adult individuals are considered. Accuracies using the scPM boundary improved by 20% for females and 16% for males but did not reach 100% for both sexes. In contrast, the scAM boundary and the P/A ratio boundary now predicted both sexes with 100% accuracy. Ratios of sex steroid concentrations were previously found effective in sexing species including Iberian wolves (Barja et al., 2008), Canada lynx (Terwissen et al., 2014), short-beaked echidnas (*Tachyglossus aculeatus*) (Oates et al., 2002), two species of turtles (Gross et al., 1995; Xia et al., 2011) and birds (Bercovitz et al., 1978; Wasser and Hunt, 2005).

The limited number of pangolin carcasses available in this study might have contributed to the lack of a significant difference in scPM and scAM levels between sexes. Despite this, the results still indicate that the use of P/A rations may be best suited to distinguish between males and females. Whole carcasses are rarely retrieved from the illegal pangolin trade and whole scales are impossible to be obtained from live individuals without hurting the animal. Therefore, a small sample size was expected for this study and will most likely remain an issue for future studies on this and other pangolin species. If a larger sample size could be achieved, significant differences in steroid concentrations between the sexes may be observed. However, the trend of clustering into a male and a female group, as well as the calculated P/A ratio and its associated boundary value are unlikely

to change substantially. Moreover, the small sample size in the present study should not take away from the fact that sex steroid concentrations could be investigated, to our knowledge, for the first time for Temminck's pangolins. Sex steroids were also previously used to approximate age class (e.g. Bautista et al., 2013; Cattet et al., 2018). In Temminck's pangolins, morphological differentiation between adult and juvenile scales based on size is difficult, as even large adults can have small scales (on the head or close to the stomach) (A. Blecher, personal observation). While juveniles in the present study differed from adults of the same sex, steroid concentrations overlapped between sexes, suggesting that age estimation may be inaccurate, but the low availability of juvenile scales warrants further investigation.

This study offers opportunities for further research including investigation of pangolin sexual maturity, scPM analysis for determination of female reproductive state and application of the sexing method for scales from other pangolin species. This is the first study to implement hormone quantification in Temminck's pangolin scales for assessing individual sex, where a ratio of scPM to scAM concentration provides the most clarity and may be employed as a forensic tool. This sexing method shows great potential for aiding forensic investigations of these trafficked mammals.

Acknowledgements:

We would like to thank SANBI for access to the pangolin carcasses and scales. Additionally, we thank Nicole Hagenah-Shrader in the Endocrine Research Laboratory of the University of Pretoria for performing the EIAs.

Funding:

We acknowledge financial support from the Rufford Foundation (grant number 28280-1) and the Pangolin Consortium.

Author statement:

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References

Ahlers, N., Creecy, J., Frankham, G., Johnson, R., Kotze, A., Linacre, A., McEwing, R., Mwale, M., Rovie-Ryan, J., Sitam, F., 2017. 'ForCyt' DNA database of wildlife species. Forensic Sci. Int. Genet. Suppl. Ser. 6, e466-e468.

Alacs, E.A., Georges, A., FitzSimmons, N.N., Robertson, J., 2010. DNA detective: a review of molecular approaches to wildlife forensics. Forensic Sci. Med. Pathol. 6(3), 180-194.

Alba, A.C., Strauch, T.A., Keisler, D.H., Wells, K.D., Kesler, D.C., 2019. Using a keratinase to degrade chicken feathers for improved extraction of glucocorticoids. Gen. Comp. Endocrinol. 270, 35-40.

Andersen, M.L., Tufik, S., 2006. Does male sexual behavior require progesterone? Brain Res. Rev. 51, 136-143.

Barja, I., Silván, G., Rosellini, S., Piñeiro, A., Illera, M., Illera, J., 2008. Quantification of sexual steroid hormones in faeces of Iberian wolf (*Canis lupus signatus*): a non-invasive sex typing method. Reprod. Domest. Anim. 43(6), 701-707.

Bautista, L.M., Silván, G., Cáceres, S., Martínez-Fernández, L., Bravo, C., Illera, J.C., Alonso, J.C., Blanco, G., 2013. Faecal sexual steroids in sex typing and endocrine status of great bustards. Eur. J. Wildl. Res. 59(6), 815-822.

Baxter-Gilbert, J.H., Riley, J.L., Mastromonaco, G.F., Litzgus, J.D., Lesbarrères, D., 2014. A novel technique to measure chronic levels of corticosterone in turtles living around a major roadway. Conserv. Physiol. 2(1), cou036.

Bercovitz, A., Czekala, N., Lasley, B., 1978. A new method of sex determination in monomorphic birds. J. Zoo Anim. Med. 9(4), 114-124.

Blecher, A.S., Scheun, J., Ganswindt, A., 2021. Degradation of Temminck's pangolin (*Smutsia temminckii*) scales with a keratinase for extraction of reproductive steroid hormones. MethodsX 8, 101229.

Bortolotti, G.R., Marchant, T.A., Blas, J., German, T., 2008. Corticosterone in feathers is a long-term, integrated measure of avian stress physiology. Funct. Ecol. 22(3), 494-500.

Brandelli, A., Daroit, D.J., Riffel, A., 2010. Biochemical features of microbial keratinases and their production and applications. Appl. Microbiol. Biotechnol. 85(6), 1735-1750.

Bräutigam, A., Howes, J., Humphreys, T., Hutton, J., 1994. Recent information on the status and utilization of African pangolins. TRAFFIC Bulletin 15(1), 15-22.

Carlitz, E.H., Kirschbaum, C., Miller, R., Rukundo, J., van Schaik, C.P., 2015. Effects of body region and time on hair cortisol concentrations in chimpanzees (*Pan troglodytes*). Gen. Comp. Endocrinol. 223, 9-15.

Cattet, M., Stenhouse, G.B., Janz, D.M., Kapronczai, L., Anne Erlenbach, J., Jansen, H.T., Nelson, O.L., Robbins, C.T., Boulanger, J., 2017. The quantification of reproductive hormones in the hair of captive adult brown bears and their application as indicators of sex and reproductive state. Conserv. Physiol. 5(1), cox032.

Cattet, M., Stenhouse, G.B., Boulanger, J., Janz, D.M., Kapronczai, L., Swenson, J.E., Zedrosser, A., 2018. Can concentrations of steroid hormones in brown bear hair reveal age class? Conserv. Physiol. 6(1), coy001.

Challender, D., Waterman, C., Baillie, J., 2014. Scaling up pangolin conservation. IUCN SSC pangolin specialist group conservation action plan, Zoological Society of London.

Challender, D.W., Heinrich, S., Shepherd, C.R., Katsis, L.K., 2020. International trade and trafficking in pangolins, 1900–2019, in: Challender, D.W., Nash, H.C., Waterman, C. (Eds.), Pangolins: Science, Society and Conservation. Academic Press, pp. 259-276.

Colding-Jørgensen, P., Hestehave, S., Abelson, K.S., Kalliokoski, O., 2020. Hair glucocorticoids are not a historical marker of stress - exploring the time-scale of corticosterone incorporation into hairs in a rat model. BioRxiv.

Davenport, M.D., Tiefenbacher, S., Lutz, C.K., Novak, M.A., Meyer, J.S., 2006. Analysis of endogenous cortisol concentrations in the hair of rhesus macaques. Gen. Comp. Endocrinol. 147(3), 255-261.

De Oliveira Martinez, J.P., Cai, G., Nachtschatt, M., Navone, L., Zhang, Z., Robins, K., Speight, R., 2020. Challenges and opportunities in identifying and characterising keratinases for value-added peptide production. Catalysts 10(2), 184.

du Toit, Z., Dalton, D.L., du Plessis, M., Jansen, R., Grobler, J.P., Kotzé, A., 2020. Isolation and characterization of 30 STRs in Temminck's ground pangolin (*Smutsia temminckii*) and potential for cross amplification in other African species. J. Genet. 99(1), 1-6.

Fox, J., Weisberg, S., 2011. Multivariate linear models in R. An R Companion to Applied Regression. Los Angeles: Thousand Oaks.

Ganswindt, A., Heistermann, M., Borragan, S., Hodges, J.K., 2002. Assessment of testicular endocrine function in captive African elephants by measurement of urinary and fecal androgens. Zoo Biol. 21(1), 27-36.

Germano, J., Molinia, F., Bishop, P., Cree, A., 2009. Urinary hormone analysis assists reproductive monitoring and sex identification of bell frogs (*Litoria raniformis*). Theriogenology 72(5), 663-671.

Germano, J., Molinia, F., Bishop, P., Bell, B., Cree, A., 2012. Urinary hormone metabolites identify sex and imply unexpected winter breeding in an endangered, subterranean-nesting frog. Gen. Comp. Endocrinol. 175(3), 464-472.

Graham, K.M., Kouba, A.J., Langhorne, C.J., Marcec, R.M., Willard, S.T., 2016. Biological sex identification in the endangered dusky gopher frog (*Lithobates sevosa*): a comparison of body size measurements, secondary sex characteristics, ultrasound imaging, and urinary hormone analysis methods. Reprod. Biol. Endocrinol. 14(1), 41.

Gregory, L.F., Schmid, J.R., 2001. Stress responses and sexing of wild Kemp's ridley sea turtles (*Lepidochelys kempii*) in the northeastern Gulf of Mexico. Gen. Comp. Endocrinol. 124(1), 66-74.

Gross, T.S., Crain, D.A., Bjorndal, K.A., Bolten, A.B., Carthy, R.R., 1995. Identification of sex in hatchling loggerhead turtles (*Caretta caretta*) by analysis of steroid concentrations in chorioallantoic/amniotic fluid. Gen. Comp. Endocrinol. 99(2), 204-210.

Guarniero, I., Mandelli, M., Stancampiano, L., Cariani, A., Govoni, N., Parmeggiani, A., Barboni, D., Mordenti, O., 2017. Genotyping, pedigree reconstruction and endocrinological characterization of *Acipenser naccarii* (Bonaparte, 1836) using microsatellite markers and plasma steroid levels. Aquac. Res. 48(11), 5550-5560.

Gupta, R., Ramnani, P., 2006. Microbial keratinases and their prospective applications: an overview. Appl. Microbiol. Biotechnol. 70(1), 21.

Gupta, S.K., 2018. Application of DNA fingerprinting and wildlife forensics, in: Dash, H.R., Shrivastava, P., Mohapatra, B.K., Das, S. (Eds.), DNA Fingerprinting: Advancements and Future Endeavors. Springer, Singapore, pp. 77-87.

Heath, M., 1992. *Manis temminckii*. Mamm. Species.(415), 1-5.

Heath, M., Coulson, I., 1997a. Preliminary studies on relocation of Cape pangolins *Manis temminckii*. SA J. Wildl. Res. 27(2), 51-56.

Heath, M., Coulson, I., 1997b. Home range size and distribution in a wild population of Cape pangolins, *Manis temminckii*, in north-west Zimbabwe. Afr. J. Ecol. 35(2), 94-109.

Hua, L., Gong, S., Wang, F., Li, W., Ge, Y., Li, X., Hou, F., 2015. Captive breeding of pangolins: current status, problems and future prospects. ZooKeys(507), 99-114.

Hunt, K.E., Stimmelmayr, R., George, C., Hanns, C., Suydam, R., Brower, H., Rolland, R.M., 2014. Baleen hormones: a novel tool for retrospective assessment of stress and reproduction in bowhead whales (*Balaena mysticetus*). Conserv. Physiol. 2(1), cou030.

The IUCN Red List of Threatened Species. Version 2020-2, 2020. The IUCN Red List of Threatened Species. Version 2020-2. Available at: https://www.iucnredlist.org. (Accessed 23 November 2020).

Johnson, R.N., Wilson-Wilde, L., Linacre, A., 2014. Current and future directions of DNA in wildlife forensic science. Forensic Sci. Int. Genet. 10, 1-11.

Kalliokoski, O., Jellestad, F.K., Murison, R., 2019. A systematic review of studies utilizing hair glucocorticoids as a measure of stress suggests the marker is more appropriate for quantifying short-term stressors. Sci. Rep. 9(1), 1-14.

Kersey, D.C., Dehnhard, M., 2014. The use of noninvasive and minimally invasive methods in endocrinology for threatened mammalian species conservation. Gen. Comp. Endocrinol. 203, 296-306.

Lattin, C.R., Reed, J.M., DesRochers, D.W., Romero, L.M., 2011. Elevated corticosterone in feathers correlates with corticosterone-induced decreased feather quality: a validation study. J. Avian. Biol. 42(3), 247-252.

Liu, Z., Jiao, D., Weng, Z., Zhang, Z., 2016. Structure and mechanical behaviors of protective armored pangolin scales and effects of hydration and orientation. J. Mech. Behav. Biomed. Mater. 56, 165-174.

Luczon, A.U., Ong, P.S., Quilang, J.P., Fontanilla, I.K.C., 2016. Determining species identity from confiscated pangolin remains using DNA barcoding. Mitochondrial DNA B 1(1), 763-766.

Mola, A.E., Hovannisyan, H.G., Ovissipour, R.M.N.M., 2011. Early sex identification in cultured beluga (*Huso huso*) using plasma steroid hormones. Afr. J. Biotechnol. 10(10), 1959-1965.

Morris, P.J., Jackintell, L.A., Alberts, A.C., 1996. Predicting the gender of subadult Komodo dragons (*Varanus komodoensis*) using two-dimensional ultrasound imaging and plasma testosterone concentration. Zoo Biol. 15(3), 341-348.

Mwale, M., Dalton, D.L., Jansen, R., de Bruyn, M., Pietersen, D., Mokgokong, P.S., Kotzé, A., 2016. Forensic application of DNA barcoding for identification of illegally traded African pangolin scales. Genome 60(3), 272-284.

Oates, J., Bradshaw, F., Bradshaw, S., Lonsdale, R., 2002. Sex identification and evidence of gonadal activity in the short-beaked echidna (*Tachyglossus aculeatus*) (Monotremata: Tachyglossidae): non-invasive analysis of faecal sex steroids. Aust. J. Zool. 50(4), 395-406.

Oettel, M., Mukhopadhyay, A., 2004. Progesterone: the forgotten hormone in men. Aging Male 7, 236-257.

Palme, R., Möstl, E., 1994. Biotin-streptavidin enzyme immunoassay for the determination of oestrogens and androgens in boar faeces, in: Görög, S. (Ed.) Advances in Steroid Analysis '93: Proceedings from the 5th Symposium on the Analysis of Steroids. Akadémiai Kiadó, Budapest, pp. 111-117.

Palme, R., 2012. Monitoring stress hormone metabolites as a useful, non-invasive tool for welfare assessment in farm animals. Anim. Welf. 21(3), 331-337.

Pietersen, D.W., 2013. Behavioural ecology and conservation biology of ground pangolins *Smutsia temminckii* in the Kalahari Desert. University of Pretoria. *Masters Thesis*.

Pietersen, D.W., McKechnie, A.E., Jansen, R., 2014. Home range, habitat selection and activity patterns of an arid-zone population of Temminck's ground pangolins, *Smutsia temminckii*. Afr. Zool. 49(2), 265-276.

Pietersen, D.W., Symes, C.T., Woodborne, S., McKechnie, A.E., Jansen, R., 2016. Diet and prey selectivity of the specialist myrmecophage, Temminck's ground pangolin. J. Zool. 298(3), 198-208.

Pietersen, D.W., Jansen, R., Swart, J., Panaino, W., Kotze, A., Rankin, P., Nebe, B., 2020. Temminck's pangolin *Smutsia temminckii* (Smuts, 1832), in: Challender, D.W., Nash, H.C., Waterman, C. (Eds.), Pangolins: Science, Society and Conservation. Academic Press, pp. 175-193.

R Core Team, 2020. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org.

Richer, R., Coulson, I., Heath, M., 1997. Foraging behaviour and ecology of the Cape pangolin (*Manis temminckii*) in north-western Zimbabwe. Afr. J. Ecol. 35(4), 361-369.

Roe, D., 2008. Trading nature: a report, with case studies, on the contribution of wildlife trade management to sustainable livelihoods and the Millennium Development Goals. TRAFFIC International & WWF International, p. 84.

Rolland, R.M., Hunt, K.E., Kraus, S.D., Wasser, S.K., 2005. Assessing reproductive status of right whales (*Eubalaena glacialis*) using fecal hormone metabolites. Gen. Comp. Endocrinol. 142(3), 308-317.

Romero, L.M., Fairhurst, G.D., 2016. Measuring corticosterone in feathers: strengths, limitations, and suggestions for the future. Comp. Biochem. Physiol A: Mol. Integr. Physiol. 202, 112-122.

Rosen, G.E., Smith, K.F., 2010. Summarizing the evidence on the international trade in illegal wildlife. Ecohealth 7(1), 24-32.

Scheffers, B.R., Oliveira, B.F., Lamb, I., Edwards, D.P., 2019. Global wildlife trade across the tree of life. Science 366(6461), 71-76.

Schwarzenberger, F., Tomášová, K., Holečková, D., Matern, B., Möstl, E., 1996. Measurement of fecal steroids in the black rhinoceros (*Diceros bicornis*) using group-specific enzyme immunoassays for 20-oxo-pregnanes. Zoo Biol. 15(2), 159-171.

Sheriff, M.J., Dantzer, B., Delehanty, B., Palme, R., Boonstra, R., 2011. Measuring stress in wildlife: techniques for quantifying glucocorticoids. Oecologia 166(4), 869-887.

Skinner, J.D., Chimimba, C.T., 2005. The Mammals of the Southern African Sub-Region. Cambridge University Press, Cape Town, South Africa.

Soewu, D.A., Sodeinde, O.A., 2015. Utilization of pangolins in Africa: fuelling factors, diversity of uses and sustainability. Int. J. Biodivers. Conserv. 7(1), 1-10.

Swart, J., Richardson, P., Ferguson, J., 1999. Ecological factors affecting the feeding behaviour of pangolins (*Manis temminckii*). J. Zool. 247(3), 281-292.

Tawichasri, P., Laopichienpong, N., Chanhome, L., Phatcharakullawarawat, R., Singchat, W., Koomgun, T., Prasongmaneerut, T., Rerkamnuaychoke, W., Sillapaprayoon, S., Muangmai, N., Suntrarachung, S., Baicharoenh, S., Peyachoknagula, S., Srikulnath, K., 2017. Using blood and non-invasive shed skin samples to identify sex of caenophidian snakes based on multiplex PCR assay. Zool. Anz. 271, 6-14.

Terwissen, C., Mastromonaco, G., Murray, D., 2014. Enzyme immunoassays as a method for quantifying hair reproductive hormones in two felid species. Conserv. Physiol. 2(1), cou044.

ul Haq, I., Akram, F., 2018. Striking applications of keratinase enzyme isolated from various natural sources: a review. Proc. Pakistan Acad. Sci. B. 55(4), 1–17.

UNODC, 2016. World wildlife crime report: trafficking in protected species. United Nations Office on Drugs and Crime (UNODC), p. 97.

Velloso, A.L., Wasser, S.K., Monfort, S.L., Dietz, J.M., 1998. Longitudinal fecal steroid excretion in maned wolves (*Chrysocyon brachyurus*). Gen. Comp. Endocrinol. 112(1), 96-107.

Wagner, C.K., 2006. The many faces of progesterone: a role in adult and developing male brain. Front. Neuroendocrinol. 27, 340-359.

Wasser, S.K., Hunt, K.E., 2005. Noninvasive measures of reproductive function and disturbance in the barred owl, great horned owl, and northern spotted owl. Ann. NY Acad. Sci. 1046(1), 109-137.

Wheeler, C.R., Novak, A.J., Wippelhauser, G.S., Sulikowski, J.A., 2016. Using circulating reproductive hormones for sex determination of Atlantic sturgeon (*Acipenser oxyrinchus oxyrinchus*) in the Saco River estuary, Maine. Conserv. Physiol. 4(1), cow059.

Witt, D.M., Young, L.J., Crews, D., 1994. Progesterone and sexual behavior in males. Psychoneuroendocrinology 19, 553-562.

Xia, Z.-R., Li, P.-P., Gu, H.-X., Fong, J.J., Zhao, E.-M., 2011. Evaluating noninvasive methods of sex identification in green sea turtle (*Chelonia mydas*) hatchlings. Chelonian Conserv. Biol. 10(1), 117-123.

Figure legends

Figure 1:

The body regions of a Temminck's pangolin (*Smutsia temminckii*) from which scales were sampled (A) and how a scale was cut (B). One scale was collected from each body region. From each scale, the base section was cut off and then cut further to obtain samples of approximately 0.40 g from the middle of the base section (indicated by the black box).Photograph of the Temminck's pangolin taken by Nigel Dennis.

Figure 2:

Median concentrations (ng/g scale dry weight (scDW)) of progestagen and androgen metabolites in scales of male (blue) and female (pink) Temminck's pangolins (*Smutsia temminckii*) of three age classes: adult (circle), sub-adult (triangle) and juvenile (rectangle).

Figure 1

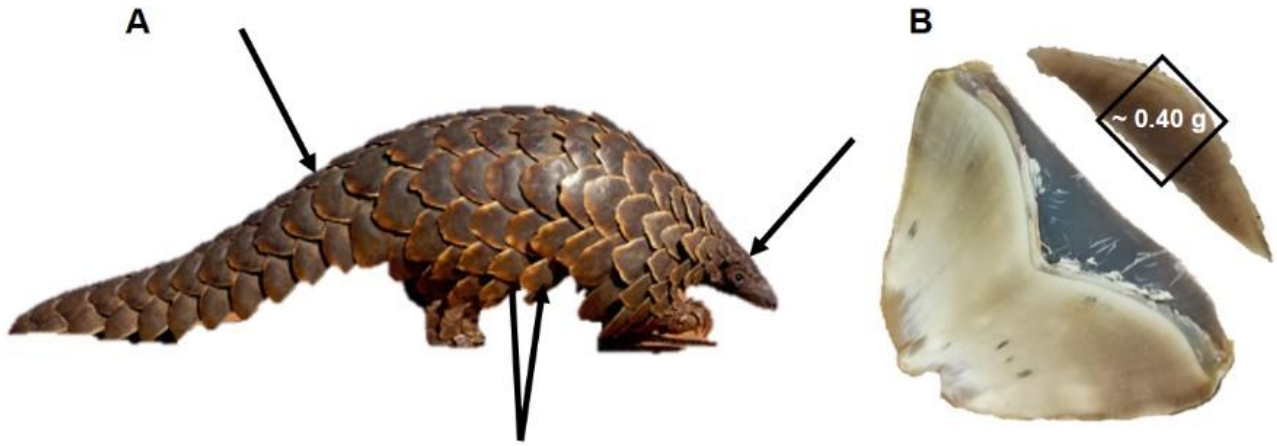

Figure 2