Pattern of faecal progestogen and oestrogen metabolite concentrations during pregnancy in Temminck's pangolin

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Preface and acknowledgements

Writing this dissertation has been a great opportunity to expand my knowledge regarding the pangolin, its reproductive endocrinology and the usage of EIA with its accompanying difficulties in application in wildlife. Between my Bachelor and Master studies in 2019, I embarked on a wildlife veterinary internship in Namibia, where I shadowed Dr. Lyndsay Scott. At R.E.S.T (Rare and Endangered Species Trust) I had my first interaction with a Temminck's pangolin and was touched by its gentle nature. It didn't take long before this interest developed further, and I grew a love and admiration for the species. After learning about the many difficulties this animal faces, its endangered status and the limited research already conducted, I wanted to use my master thesis to help its conservation by improving the scientific knowledge on its procreation.

The trajectory of this thesis has been anything but smooth sailing and would have been impossible without the indispensable help and support of several people.

First and foremost, I want to thank my promotor, Prof. Dr. Jella Wauters for having the patience of guiding me along the difficult path of writing a master thesis. Especially considering her busy schedule, I am grateful for her time and effort spent reviewing my writing, guiding me through the permit applications and providing the ethics permit. She made me realize that studying veterinary sciences is still something entirely different from writing a valid academic paper.

As the habitat of the object of my study, the Temminck's pangolin, is Southern and Eastern Africa I am very grateful that I could rely on my co-promotor: Dr. Karin Lourens (co-founder and veterinary surgeon), Nicci wright (co-founder and wildlife rehabilitation specialist) and the other employees and volunteers of the Johannesburg Wildlife Veterinary Hospital, to gather the necessary faecal samples over an extensive period of time (from April 2021 to June 2022).
Even though practical conditions were not always favourable, they showed dedication and tenacity in following, for which I am truly indebted. Without them this research would not have been possible. I also want to thank Dr. Karin Lourens for using her extensive knowledge on the pangolin in reviewing some of my writing, and Nicci Wright for arranging the practical aspects of sample collection and keeping me updated in this process.

And last but not least Prof. Dr. Andre Ganswindt was so kind to offer the services of his laboratory at the Mammal Research Institute at Pretoria University to develop and perform the necessary assays. Because of Covid I was unfortunately not able to travel to South Africa to assist in the analysis, as was my initial outset. Otherwise I could have made more use of his expertise in endocrinology. I'd also like to thank Dr. Andrea Webster, postdoctoral researcher at the institute for her guidance and help in obtaining the section 20 permit.

Finally, I want to thank my family and friends, and especially my mother. Not only did she support me through the six years of veterinary studies, she also helped me overcome many obstacles.

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Abbreviations

11b-HSD: 11b-hydroxysteroid dehydrogenase
ACTH: Adrenocorticotropin hormone
AP: Alkaline phosphatase
AVP: Arginine Vasopressin
BSA: Bovine Serum Albumin
CBD: Convention on Biological Diversity
CBG: Corticosteroid binding globulin
CITES: The Convention on International Trade in Endangered Species of Wild Fauna and Flora
CNS: Central nervous system
CoP: Conference of the Parties to CITES
CRH: Corticotropin releasing hormone
CV: Coefficient of variation
DHEA : Dehydroepiandosterone
EIA: Enzyme immune assay
fEM: Faecal oestrogen metabolites
fPM: Faecal progestogen metabolites
GC: Glucocorticoids
GnRH: Gonadotropin Releasing Hormone
GO: Glucose oxidase
GR: Glucocorticoid receptors
GRE: Glucocorticoid response elements
HPA: Hypothalamus- pituitary- adrenal axis
HPG: Hypothalamus-anterior pituitary- peripheral gonadal gland axis
HRP: Horse radish peroxidase
HS-BSA: High sensitive Bovine Serum Albumin
IUCN: International Union for Conservation of Nature
JWVH: Johannesburg Wildlife Veterinary Hospital
Lag time: delay between hormone secretion and excretion
LC-NE: Locus coeruleus-norepinephrine
LD: Limit of detection
LLOQ: Lower limit of quantification
MR: Mineralocorticoid receptors
RSD: Relative standard deviation
SA: South Africa
TOPS: Threatened or Protected Species
ULOQ: Upper limit of quantification

1. Abstract

The Temminck's pangolin (*Smutsia temminckii*), is one of eight species belonging to the order Pholidota. They are predominantly nocturnal mammals, surrounded and protected by their distinctive keratinized scales. Being myrmecophagous, the Temminck's pangolin will only consume ants and termites. Despite being a relatively unknown species, pangolins face many threats, hunting, electrocution, and especially the illegal wildlife trade of their keratin scales (for traditional medicinal purposes) have driven the decline off all pangolin species globally. Currently named critically endangered by the IUCN and CITES, well developed *In situ* and *Ex situ* conservation plans are urgently needed to aid this species.
Due to its elusive nature and relative unfamiliarity, the Temminck's pangolin is a severely understudied species, especially in terms of its reproductive knowledge. Many parameters, including the oestrus cycle, and hormone concentrations have not been investigated yet. Others such as the seasonality and gestation period, have too much variation in their reported data, indicating the need for further research. To improve our knowledge and aid conservation implementation, this research aim is to establish a non-invasive protocol for monitoring faecal progestogen and oestrogen metabolites in wild female Temminck's pangolins (smutsia temmincki). A protocol and study setup will be made, and validated, through which we hope to identify the principal faecal oestrogen and progestogen metabolites, as well as clarify the oestrus cycle. Considering the endangered status, biological validation will happen through comparison of pregnant and nonpregnant animals. Unfortunately, because of numerous difficulties faced during the collecting and transportation of the samples, results could not be analysed on time for this dissertation, and will be published later.

Samenvatting

Het Temminck's schubdier (Smutsia temminckii) is één van de acht soorten die behoren tot de orde Pholidota. Het zijn overwegend nachtactieve zoogdieren, omgeven en beschermd door hun typische keratine schubben. Omdat het schubdier myrmecofaag is, eet het alleen mieren en termieten. Ondanks het feit dat het een relatief onbekende soort is, worden schubdieren met vele bedreigingen geconfronteerd: jacht, elektrocutie en vooral de illegale handel in hun schubben (voor traditioneel medicinaal gebruik) hebben wereldwijd geleid tot een daling van alle schubdierspecies. Deze soort wordt door de IUCN en CITES verklaard als een ernstig bedreigde diersoort, waarvoor er een dringende nood is aan goed ontwikkelde in situ en ex situ conservatieplannen.
Door zijn schuchtere aard en relatieve onbekendheid is het Temmincks schubdier weinig bestudeerd geweest, vooral op het vlak van voortplanting. Vele parameters, zoals de oestruscyclus en hormoonconcentraties, zijn nog niet onderzocht. Andere, zoals de seizoensgebondenheid en de draagtijd, vertonen te veel variatie in de gepubliceerde data, dit wijst op de noodzaak van verder onderzoek. Om onze kennis te verbeteren en het behoud van de soort te bevorderen, zal dit onderzoek gericht zijn op het opstellen van een niet-invasief protocol voor het monitoren van progestageen- en oestrogeenmetabolieten in de feces van wilde vrouwelijke Temminck's schubdieren (smutsia temmincki). Een protocol en studieopzet hiervoor, zullen worden gemaakt en gevalideerd, waarmee we hopen de voornaamste fecale oestrogeen- en progestogeenmetabolieten te identificeren, alsook de oestruscyclus te verduidelijken. Gezien de bedreigde status, zal biologische validatie gebeuren door vergelijking van drachtige met niet-drachtige dieren. Jammer genoeg konden, wegens talrijke moeilijkheden bij het verzamelen en vervoeren van de stalen, de resultaten niet tijdig geanalyseerd worden voor dit proefschrift, en zullen deze later gepubliceerd worden.

2. Introduction

2.1   The Temminck's pangolin

The pangolin, also known as "scaly anteater", received this nickname due to the large overlapping keratin scales, that cover most of its body. When the animal rolls up into a tight ball, these nearly impenetrable scales form a shield against predators, and protect its delicate abdomen. The scales also guard them against scratches from underbrush and rocks during foraging (Heath, 1992; Pietersen et al., 2019).

Pangolins are myrmecophagous (i.e. only consuming termites and ants). They are often highly selective in the species they prey upon. It is estimated that adults can consume more than 70 million ants and termites annually. Because of this, they are suspected to play an important ecological role in the regulation of these populations. Diet composition differs with different latitudes, seasonal changes and between pangolin species. The Temminck's pangolin's food includes 15 species of ants and five species of termites (Pietersen et al., 2019; Durojaye and Olufemi, 2015; Hua et al., 2015; Heath, 1992). When foraging, which can last up to six hours, the Temminck's pangolin puts most of its weight on the hind legs, as such being the only bipedal pangolin species whilst using its tail for counterbalance. Upon localisation of the prey, they use the three central front claws, which can be up to 5 cm long, to tear open ant and termite nests. They feed in short bouts to prevent complete exhaustion of the colony (Pietersen et al., 2019; Heath, 1992). Adapted to their dietary preferences, pangolins lack teeth and have a delicate jaw with a small mouth opening, resorting on their tongue for feeding. The long tongue can extend up to 30-40 cm outside of the mouth, and has a total length of 40-60 cm, attaching to the caudal end of the cartilaginous xiphisternum. Glue-like saliva helps them take up the prey while accompanying grit and sand, together with the muscular movement, assists in the grinding process (Pietersen et al., 2019; Totton, 2011).

In general, pangolins are solitary and nocturnal creatures (Hua et al., 2015; Totton, 2011; Heath, 1992). However, Pietersen et al. (2019), suggests that some individuals may be active during the twilight period, whilst others show a diurnal pattern during winter months. Lim and Ng (2008) found similar diurnal behaviour in a female Sunda pangolin (*Manis javanica)*, with her young, during the month of December. This behaviour displayed by mother and young, happened in the last phase of maternal care. Being nocturnal, pangolins have adapted their eyesight to darkness. They have a wide corneal diameter, which increases their sensitivity to light, but also makes their vision less sharp. Although pangolins don't have poor eyesight, this does suggest less reliance on vision and more reliance on their other senses i.e. their well-developed olfactory sense (Adekanmbi and Akinola, 2017).

2.1.1   Phylogeny and demographic distribution

The pangolin is a mammal classified within the order Pholidota, family *Manidae*. In the *Manidae* family there are eight extant species (four Asian species and four African species). Through the use of whole mitochondrial DNA genomes, it has been shown that the African and Asian pangolin species are two distinct monophyletic clades, both groups descending from a different common ancestor, as seen in figure 1. Based on morphological and genetic evidence, the *Manidae* family further specifies into three genera: *Manis* (*M. crassicaudata, M.culionensis, M.javanica, M.pentadactyla*) to which the four Asian species belong, *Phataginus* (*P. tetradactyla, P. tricuspis*) corresponding to the arboreal African pangolins and *Smutsia* (*S. gigantea, S. temminckii*) which are the ground-dwelling African pangolins. Another study, using whole mitochondrial DNA, made an interesting discovery. Pholidota and Carnivora, both members of the Laurasiatheria clade, share a common ancestor 87 million years ago, forming a monophyletic group. The discovery of the pangolin being more closely related to the Carnivora than to the phenotypically more similar order Xenarthra (to which the nine-banded armadillo and lesser anteater belong) provides new insight and opportunities when studying pangolins in the future (Du Toit et al., 2017, 2014; Pietersen et al., 2019; Totton, 2011).
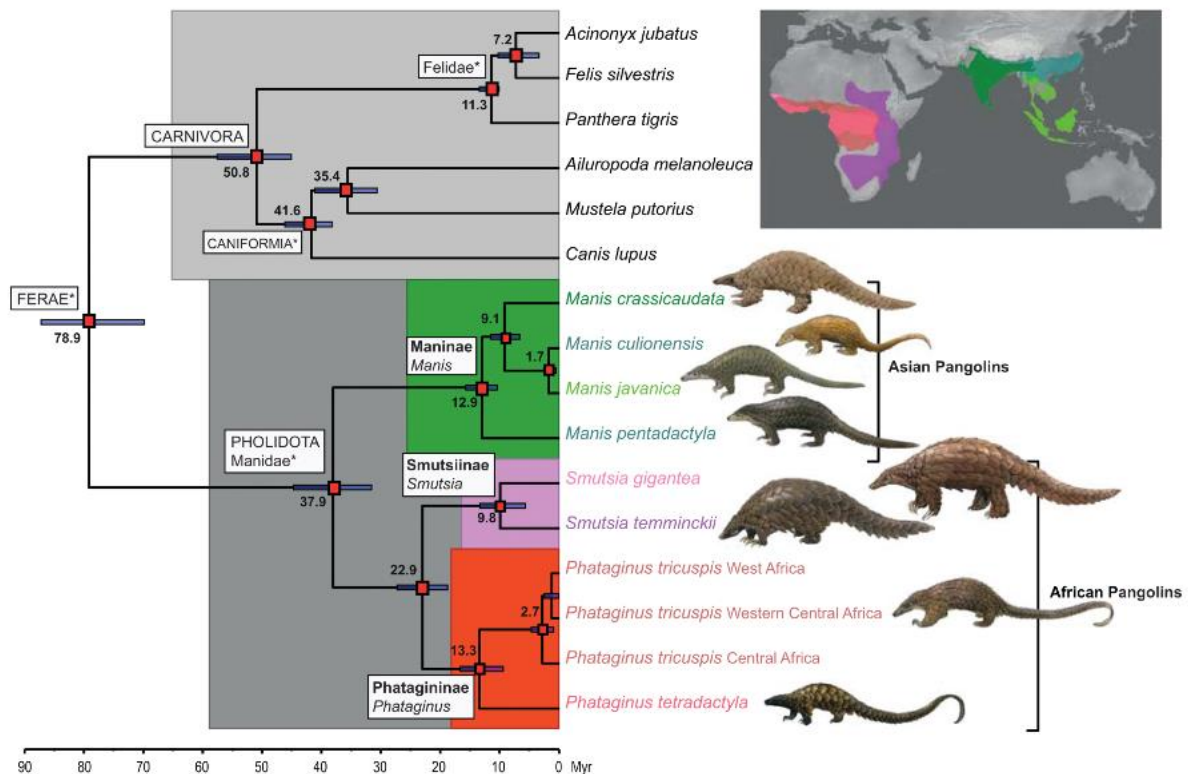
Figure 1: Tree of extant pangolin species, and most recent common ancestors with the carnivora order (from: Gaubert et al., 2018).

The Temminck's pangolin (*Smutsia temminckii)* also known as the South African pangolin, ground pangolin or Cape pangolin (Zondi, 2018)[1], can be found throughout most of Southern and Eastern Africa, but is absent from Saharan and Western Africa. They may inhabit savannah, woodland, floodplains, bush willow, and Duneveld grassland but cannot be found in closed-canopy forests or true desert. Their distribution varies and is mainly determined by the available prey species (Pietersen et al., 2019; Heath, 1992). Temminck's pangolins have home ranges that are adjacent to each other, with a slight overlap sometimes observed. The home ranges are estimated between 0,17 to 11,07 km, with male pangolins and older pangolins occupying larger areas (Heath and Coulson, 1997). Within their specified home range, they utilise abandoned burrows as a temporary den, moving to another one after a certain time (Pietersen et al., 2019).

2.1.2   Reproductive biology

Female pangolins have two pectoral mammae of about 1cm long and a bicornuate uterus. They give birth to one young, referred to as a pup, every year or possibly every other year (Chin et al., 2012; Heath, 1992; Zhang et al., 2015). On the other hand, for the Indian and Chinese pangolin, reports about two pups have been made (Zhang et al., 2016). Breeding appears to be unseasonal but a seasonal peak of late summer to early autumn has been suggested (Pietersen et al., 2019; Heath, 1992). Hua et al. (2015) mentions "Female pangolins have two to five estrus cycles during the mating season, and each will last for 11-26 days, until conception occurs." They have a gestation period estimated between 105-140 days. Considering very little research is published on the reproductive parameters of pangolins, especially on the Temminck's pangolin, this data is most likely obtained from research on the Chinese Pangolin (Pietersen et al., 2019; Hua et al., 2015; Heath, 1992). Details on the reproduction cycle, such

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[1]   See: Zondi, Z., 2016. Temminck's Ground Pangolin. https://www.sanbi.org/animal-of-the-week/temmincks-ground-pangolin/. (accessed 5.17.2021).

as duration, follicular and luteal phase length are currently no reports on. All pangolin species have an epitheliochorial placental type (Carter and Enders, 2004).

The post-partum care is not well documented yet and reports vary. It is mentioned that the female will carry the juvenile on her back and will curl around the juvenile when threatened. The young becomes independent between 3 to 12 months, but the latter information varies depending on the source, e.g. ranging from six months to one year for the Sunda pangolin (M.*Javanica*) (Zhang et al., 2015) and between one and one and a half year for the Formosan pangolin (M.*pentadactyla*)(Chin et al., 2012). The variation in reported data on reproductive parameters and the absence of scientific data regarding the estrus cycle, gestation time, gonad activity etc., indicates the need for more research in this area and on the different species (Pietersen et al., 2019; Wickers et al., 2019).

Currently information on the reproduction parameters of the temminck's pangolin and pangolins in general is scarce. Many parameters, including the oestrus cycle, cyclicity and hormone concentrations have not been investigated yet. Others such as the seasonality and gestation period, have too much variation in their reported data. This indicates the need for further research.

2.2   Illegal trade and other threats

Numerous anthropogenic threats are driving the decline of the Temminck's pangolin. Accidental electrocution on electric fences in South Africa (SA) kills between 377-1028 pangolins annually. Which, based on the population median estimate of Pietersen et al. (2016), comprises 3,5% of the South African Temminck's population. This high number may be correlated to the substantial number of electric fences used in game reserves in SA (obliged by law), and the fact that the Temminck's pangolin walks more upright than other species, exposing the unprotected belly when passing the bottom trip wire on these electric fences. Fortunately, some reserves are trying to lessen the impact of the electric fences by altering them and making it safer for smaller species, including the pangolin. Other threats are habitat loss, capture in snares, (accidental) poisoning and roadkill. Traffic in Namibia and South Africa cause the estimated death of 280 pangolins annually (Pietersen et al., 2019; Wright et al., 2019; Pietersen et al., 2014).

Although prohibited, hunting and bush meat extraction in Africa is exceptionally high. Pangolins are not only hunted for their meat but also for their body parts and scales. This due to superstitious beliefs and their use in traditional medicine. Interviews of traditional practitioners in Ghana and Sierra Leone showed that numerous pangolin body parts are used for a wide range of ailments, but the most popular parts are the scales and bones. They are believed to treat spiritual ailments, rheumatism, infertility, convulsions, skin disease and other health issues. In Ghana traditional medicine is estimated to be used by 80% of the population and is recognized by the government as a component of the healthcare delivery system (Yisau et al., 2019; Pietersen et al., 2015; Boakye et al., 2015, 2014).

The use of pangolins, mainly their scales, is also common in many parts of Asia. Because of the decline in Asian pangolin populations, the local supply is unable to meet the increasing demand. The latter, as well as the growing economic ties between the two continents, play a crucial role in the increase of the illegal, international trade of Temminck's pangolins from African countries to Asia (Pietersen et al., 2019; Heinrich et al.,
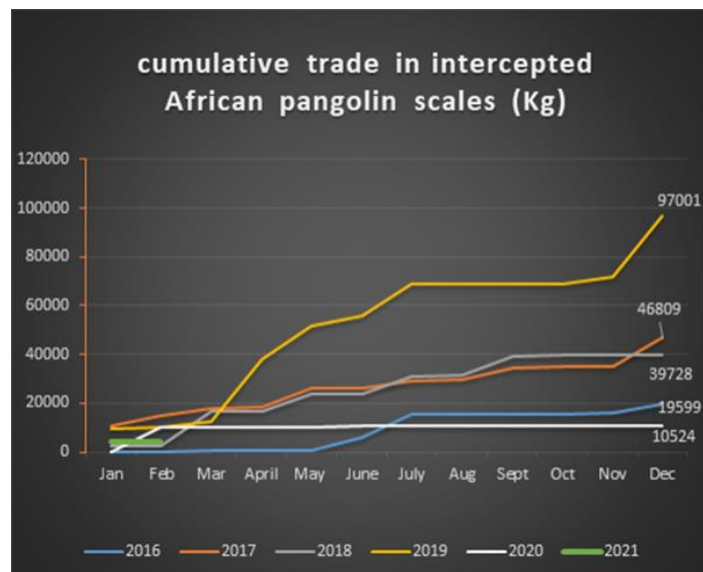
Figure 2: Interceptions in African pangolin scale trade, destined for or retrieved in Asian countries. Data ranging from 2016 until the beginning of 2021 *(from APWG, unpublished data).*

2016). This is demonstrated by the graph in figure 2, which shows the growing illegal trade in African pangolin scales over the past 5 years (African Pangolin Working Group, unpublished data, January 2022).

Trade in pangolin species is a global phenomenon and a total of 1485 illegal trade incidents have been reported to CITES, With an estimated 809,723 whole pangolins involved in the trade for the entire period between 1977 and 2014 (Pietersen et al., 2019; Heinrich et al., 2016).

Swift et al. (2007) states that: "The trade in wildlife not only threatens the integrity of ecosystems worldwide, but also poses a serious and increasing risk of **initiating epidemics** of emergent infectious diseases in human populations." Through a risk assessment model, they showed that the hunting pressure and change in hunter-capture profiles could lead to an increased risk of outbreaks. The change from rural to urban settings, containing a higher concentration of people with lower levels of immunity, further increases the probability of epidemic spread. Several *Amblyomma* tick species have been found throughout the species of pangolins, these ticks have been recorded to also feed on humans, serving as potential vectors for transmission of disease (e.g., African tick bite fever, Ehrlichiosis). They may also serve as reservoirs for *Trypanosoma*, causing sleeping sickness, Chagas disease and toxoplasmosis. Recently two novel RNA viruses were identified from four sick pangolins in China. These viruses where not found in the local tick population and might have been imported following the illegal international trade of pangolins. The latter illustrates that epizootic pathogens pose a major threat to humans and wildlife species, particularly in the context of changing environments (Gao et al., 2020; Mohapatra et al., 2020, 2016).

The growing illegal trade and numerous threats have caused a serious decline in pangolin numbers. Apart from proper legislation and implementation, well developed conservation plans are necessary to aid this species' survival

2.3   Conservation

2.3.1 Conservation status

The low rate of reproduction combined with the rapid increase in pangolin poaching, cause pangolin numbers to decline at an alarming rate (Yisau et al., 2019). Pietersen et al. (2016) estimated a "decline of 30%, over a 27 year period, and based on the IUCN red list of threatened species[2], all eight pangolin species face a high risk of extinction in the wild. This listing is based on a conservative assessment as accurately determining their numbers in the wild is nearly impossible, but nevertheless gives the best indication of a species' conservation status. Three of the Asian pangolin species are listed as critically endangered, the Indian pangolin (*Manis crassicaudat)* and two of the African pangolin species are listed as endangered while the Temmicks's pangolin and black bellied pangolin are classified as vulnerable (Hoffmann and Challender, 2019; Challender et al., 2019[2]). The 17th meeting of the Conference of the Parties to CITES (CoP, in 2016), proved to be a key moment for pangolins. In the CoP17 there was almost unanimous support for the proposal to transfer all pangolins species from appendix two to one (Appendix one including species who are threatened with extinction), which has ensured the global trade of pangolins being banned, as from January 2017. CoP17 also urged parties to ensure that legislation and penalties are in place, to undertake capacity building activities, to put adequate control measures in place for stockpiles, to partner up with local communities, to develop *in situ* monitoring of pangolin populations and to implement measures to reduce the demand (Challender and O'Criodain, 2019; Thomson and Fletcher, 2019; du Toit et al., 2017).

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[2] Challender, D., O'Neill, H., Willis, J., 2019. IUCN Red List update highlights need for concerted conservation action for pangolins [WWW Document]. URL https://www.pangolinsg.org/2019/12/23/iucn-red-list-update-highlights-need-for-concerted-conservation-action-for-pangolins/ (accessed 4.20.2021).

2.3.2 Conservation programs

Because of the various threats, such as increasing illegal trade with impending risk of extinction, it was essential to develop conservation strategies on international, national, and regional levels. Regulations on pangolin trafficking and law enforcement play a crucial role in this. There are two relevant international instruments: **The convention on Biological Diversity** of 1992 (CBD) and CITES. The CBD covers the use and conservation of biodiversity. However, this convention does not lend itself to clear, specific, and uniform implementation. **CITES,** which targets sustainability in international wildlife trade, has precise requirements for implementation and clear legal terms to guide this. All pangolin range states are members of CITES, though only 55% have legislation that meet the requirement for implementation. Pangolins are well protected on paper, but lack of implementation and enforcement of these regulations cause low rates of success. Front line enforcement agents and judiciaries with a greater knowledge of wildlife crime, better protection at borders, international cooperation, dedicated intelligence officers, a rapid response system and community engagements are some elements that could improve effective law enforcement. Credibility and trust between law enforcement officers and the wider community provides a way for the community to exercise their voice and brings engagement in conservation and information about traffickers (Challender et al., 2019; Cooney et al., 2019; Harrop, 2019; Plowman, 2019). An example of this is the Zimbabwean approach where pangolin related crime is viewed as a serious wildlife offence and a person convicted is liable to imprisonment for 9 years or a fine. The Zimbabwean government also produced a handbook for evaluating and prosecuting wildlife crimes (Shepherd et al., 2017).

Most species conservation strategies or action plans have been developed in association with, or by International Union for Conservation of Nature **(IUCN)**. In 2008, the IUCN handbook for strategic planning for species conversation was published, allowing for the joint development of conservation strategies by all parties involved. Within the IUCN, a pangolin specialist group was formed, formulating objectives based on the three components of the "Species Conservation Cycle": assessing the status, conservation planning, facilitating conservation strategies and actions. Some projects being undertaken by Pangolin Specialist Group members for the Temminck's pangolin include a) the establishment of the African pangolin working group who monitors pangolins post release, survival, and habitat use. This group is also working on counter poaching, with trained pangolin detection dogs (Counter-poaching K9 pangolin detection programme) and the reintroduction of pangolins into locally previously extinct ranges. b) the instalment of rescue, rehabilitation, and release Centres. These include the Tikki Hywood foundation in Zimbabwe, NARREC in Namibia and the Johannesburg wildlife veterinary hospital (JWVH) in South Africa, the latter combining a registered veterinary practice for treating compromised pangolins with a rehabilitation facility. c) the development of IUCN Ex Situ Guidelines. These contain a five-step decision making process to ensure that the tools of *ex situ* conservation complement and do not undermine *in situ* conservation (IUCN, 2021; Challender et al., 2019; Hoffmann and Challender, 2019; Parker and Luz, 2019).

Conservation programs usually thus consist of an *ex situ* and *In situ* part. *Ex situ* efforts support conservation outside of the animals' natural habitat, whereas *in situ* efforts are focused on maintaining and expanding the natural habitat of the animal and/or reintroduction/translocation of individuals. Some *ex situ* initiatives include support of conservation and research programs, raising awareness, ensure captive back up populations and rescue-rehabilitation and reintroduction of wildlife. *In situ* conservationists establish conservation reserves, increase community knowledge, and tackle local problems. Both approaches are different but both essential and complementary in a good conservation program.

Collaboration between the conservation stakeholders and the local community is imperative to create an effective conservation plan (Parker and Luz, 2019; Maxted, 2013). In the development of a conservation plan it is important to discuss which *ex situ* activities will be beneficial to the species and to weigh the potential benefits against the costs and risks. *Ex situ* conservation through raising awareness, research projects, and financial support is proven to have a positive impact on conservation. For pangolins no consensus has been reached about the relevance of maintaining a captive back-up population, mainly due to the many challenges associated with properly managing a healthy population of this species in captivity. Pangolins have a very specific diet, frequently occurring health issues and

high susceptibility to stress, therefore survival rates are poor in captivity. Consequently, *Ex situ* long term management of captive pangolins, might inadvertently contribute to overexploitation and jeopardize the wildlife population.

For this reason, a better alternative might be for conservation zoos to work closely with approved rescue, rehabilitation, and release centres such as the Tikki Hywood foundation in Zimbabwe, NARREC in Namibia, the Save Vietnam's Wildlife in Vietnam, and the Johannesburg wildlife veterinary hospital in South Africa. These centres make an important contribution to the conservation of the species by offering a lifeline to rescued animals (Parker and Luz, 2019; Wright and Jimerson, 2019; Wicker et al., 2019). They provide intensive supportive care to pangolins who are injured and/or confiscated from the illegal trade, until they are strong enough for release/reintroduction. In South African centres, once the Temminck's pangolin is fit enough, he is taken on foraging excursions to consume his natural diet of ants and termites. The Temminck's pangolin does not consume a captive diet and the foraging excursions minimize stress and allow for normal behaviour to occur. Decisions on release are also often made with a long-term view, as keeping the pangolins in captivity longer may result in progressing health problems (Wicker et al., 2019). In South Africa, an increasing number of live Temminck's pangolins get confiscated every year (NDPP, 2018), some of these might be pregnant. Due to lack of research regarding the reproductive biology of pangolins, more specifically the Temminck's pangolin, correct recognition of pregnancy cannot always be made. The Temminck's pangolin already has a low rehabilitation-recovery success rate (Meyer, 2020) and a low reproduction rate (Lim and Ng, 2008), making the survival of rescued pregnant females even more important. Gaining knowledge about their reproductive biology, oestrus cycle and establishing a non-invasive method for pregnancy detection, can aid *in situ* conservationists in developing a pregnancy-specific management protocol. Knowing an animal's reproductive status allows rehabilitators to adjust their veterinary care, feeding, housing, etc. accordingly. Pregnancy detection will, however, only provide the conservationist with knowledge about the pregnancy status. More research might be needed, regarding medication usage, stress-minimalization, feeding and optimal care of pregnant pangolins.

2.4 Reproduction

Proper diagnosis of pregnancy requires knowledge of the reproduction steroids, the oestrus cycle and its corresponding fluctuations during pregnancy.

2.4.2 Steroid metabolism

Steroids are small, naturally occurring, important signalling molecules. Tiny structural changes can result in big functional changes. All steroids derivate from a fused hydrocarbon ring structure, containing three six membered carbon rings and one 5-membered carbon ring (figure 3) (Wudy et al., 2018). Classification can happen based on the substituents at specific sites of the hydrocarbon structure, classifying them into androstane-, pregnane- or estrone ring structures. Another way, used more frequently, is the classification based on their biological activities, which divides the steroids into androgens, oestrogen, progestogen, glucocorticosteroids (cortisol), mineral corticoids (aldosterone), vitamin D (1α,25(OH)2D3) and bile acids (Norman and Henry, 2015a; Wudy et al., 2018).

Figure 3: Cholesterol structure (from: wudy et al., 2018.)
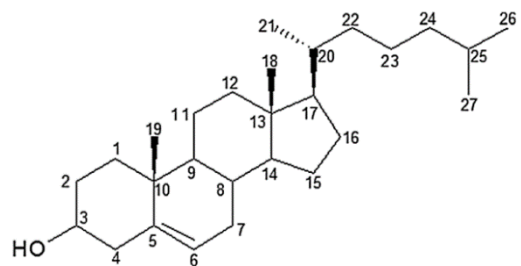
The steroid hormones in all mammals are derived from cholesterol, and follow a similar pathway, shown in figure 4. Each steroid hormone is produced in a specific endocrine gland, after stimulation by their respective tropic (stimulatory) hormone. They are mainly synthesised in the gonads (androgens, oestrogen and progestogens) or in the adrenal gland (mineralocorticoids, corticosteroids and androgens) (Norman and Henry, 2015a). Steroids can travel through the bloodstream in a bound or unbound form. The majority is bound to their respective plasma transport proteins, synthesised in the liver. For example, corticosteroids are bound to corticosteroid binding globulin (CBG). Bound steroids

cannot leave the bloodstream, therefore they must first dissociate in able to diffuse through the cell membrane of target cells (Andrew, 2001; Norman and Henry, 2015a; Silva et al., 2017).
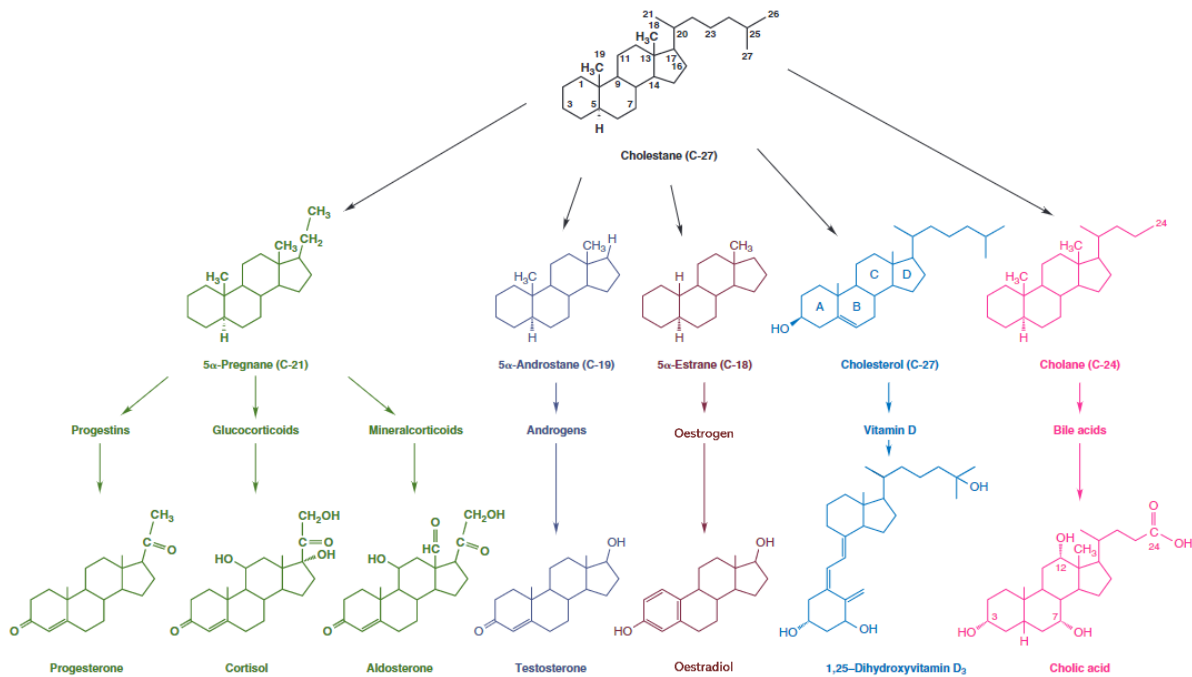

Figure 4 : Classification of the seven classes of steroids, structurally derived from cholestane. Cholestane is the parent ring structure of cholesterol (from: Norman and Henry, 2015).

After exerting their various effects, steroids are metabolized and inactivated by the liver and kidney and then excreted in bile and/or urine. Steroids then pass through the intestinal tract in which they can be re-absorbed into the entero-hepatic circulation or undergo alteration by the intestinal microbiome. This passage creates a lag-time (time between circulation in plasma and appearance in faeces); which ensures less interference of daily rhythm and acute stress but is important to consider when interpreting and linking the results to physiological events. The lag time also varies between species. Depending on the transit time of the intestinal tract, this can range from 12 hours to more than two days (Kleiman et al., 2010; Palme, 2019; Peter et al., 2018; Schwarzenberger et al., 1996; Sheriff et al., 2011; Volpato, 1999).

2.4.3   Sex steroids

Oestradiol and progesterone are the two most important female steroids. These sex steroids get mainly formed, locally, in the female reproductive tissues (ovary, placenta). Oestrogen is synthesised by the granulosa and theca cells of the ovaries, and a bit by the corpus luteum and placenta. Progesterone is produced by the corpus luteum and the placenta. High progesterone levels are essential for the maintenance of early pregnancy. Cholesterol gets converted into pregnenolone and then into progesterone and dehydroepiandrosterone (DHEA), as shown in figure 5. DHEA then follows a major or minor pathway, ultimately forming 17β−oestradiol, which is the biologically active form in mammals (Norman and Henry, 2015a).

The main regulator of sex steroids, gonadal activity, and ovulation is the hypothalamus-anterior pituitary-peripheral gonadal gland axis (HPG). The tonic centre and the surge centre, both located in the hypothalamus, produce Gonadotropin Releasing Hormone (GnRH). GnRH then regulates the secretion of Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) from the anterior pituitary. FSH stimulates the follicle formation and the secretion of a gonadal produced peptide hormone: inhibin. LH stimulates the production of progesterone by the corpus luteum, and together with FSH the production

of oestradiol. Oestradiol production requires the co-operation of both theca- and granulosa cells in the ripening follicles and LH and FSH. The tonic centre produces a low basal level of GnRH, this happens in a pulsatile manner. Regulation happens through negative feedback loops e.g. production of inhibin which inhibits the secretion of FSH. Increased concentrations of oestrogen, progesterone, FSH and LH have also an inhibitory effect on the secretion of GnRH (Norman and Henry, 2015b; Sjaastad et al., 2016).

When the ripening follicles' oestrogen production passes a certain threshold, the neurons in the surge centre of the hypothalamus release GnRH, followed by a corresponding peak in LH and FSH. FSH however is inhibited by inhibin, resulting in solely a LH peak. This LH peak is needed for ovulation (Sjaastad et al., 2016).
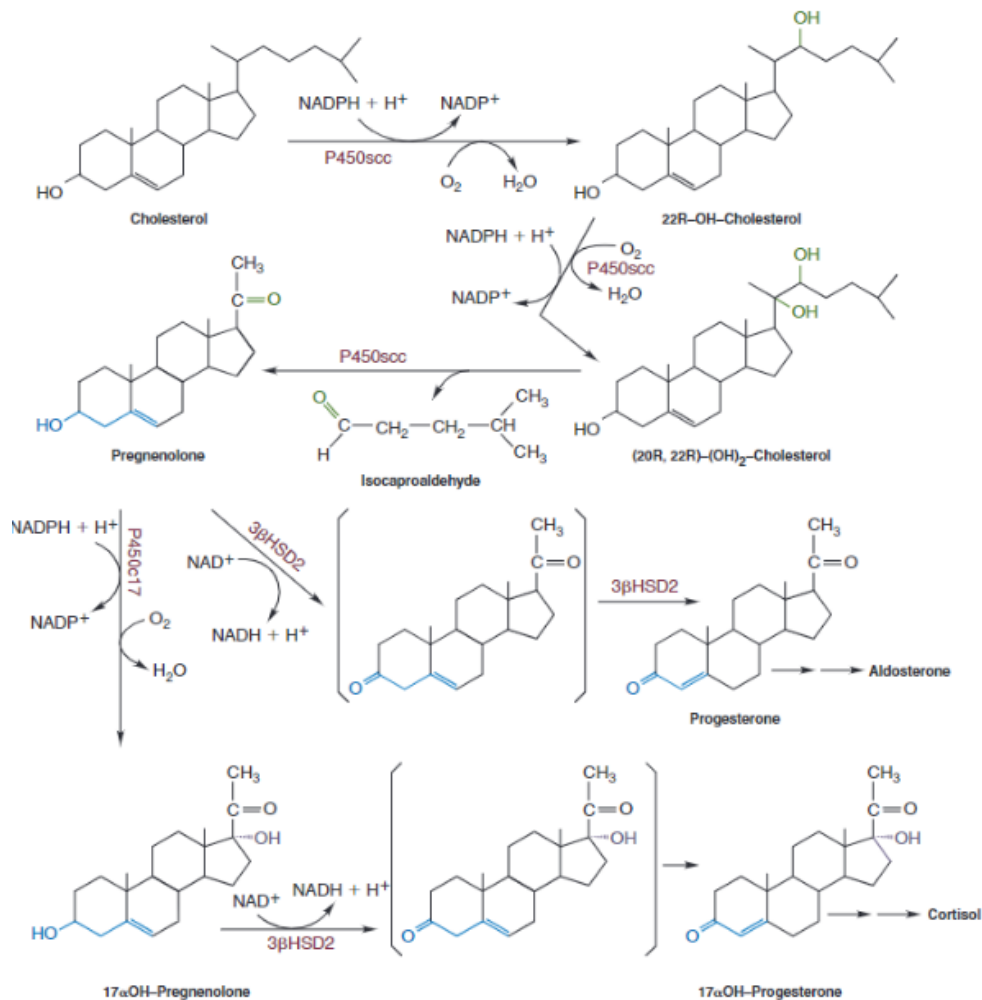
Figure 5: Production of sex steroids (progesterone and oestradiol). Cholesterol being the parent hormone of both (from: Norman and Henry, 2015).

After executing their effects, steroids are metabolized by the liver and excreted in the urine (Andrew, 2001; Bras Zootec and Schwarzenberger, 2007). Oestrogens are often found unchanged in faeces, with the principle oestrogen metabolites being estrone, oestradiol-17α and oestradiol-17β (Schwarzenberger, 2007; Schwarzenberger et al., 1996; Silva et al., 2017; Volpato, 1999). Studies show that oestradiol-17β is the main oestrogen metabolite in many species, including the Beira Antelope (Dorcatragus *megalotis*) (Wolf et al., 2019), and the snow leopard (Panthera *uncia*) (Kinoshita et al., 2011). In contrast to oestrogen, progesterone is often metabolised before excretion. Umapathy et al. (2013) showed that: "in faecal samples of most big cats, progesterone is excreted as 5 alpha and 5 beta reduced pregnanes." This is in agreement with Schwarzenberger et al. (1996), who reported that this is the major progesterone metabolite in many species.

2.4.4 Glucocorticoids

The primary glucocorticoid produced, varies between species, but most mammals secrete primarily cortisol. In some species both cortisol and corticosterone are present, in which cortisol has a greater biological activity then corticosterone. Under 'normal' (no stress) circumstances, cortisol secretion follows a circadian and pulsatile rhythm, with amplitude changes seen in the morning (Charmandari et al., 2005; Silva et al., 2017). Cortisol, also called "the stress hormone" is an essential component in the stress response. It regulates metabolic activity, behaviour, physiological- neuroendocrine responses and reproduction (Palme, 2019; Whirledge and Cidlowski, 2017).

The most important regulators of cortisol are the sympathetic adrenomedullary system and the Hypothalamus- pituitary- adrenal axis (HPA axis) (Sheriff et al., 2011). The hypothalamus primarily regulates this through the production of Corticotropin releasing hormone (CRH) from the paraventricular nucleus (PVN), which then via the hypophyseal portal system, stimulates the secretion of adrenocorticotropin hormone (ACTH) from the anterior pituitary. The hypothalamus also secretes arginine vasopressin (AVP), which has a synergistic effect on CRH. Through the secretion of ACTH, the anterior pituitary then in turn stimulates glucocorticoid secretion from the zona fasciculata of the adrenal cortex. The glucocorticoids bind to glucocorticoid receptors (GR) in the cytoplasm of ubiquitously distributed target cells, and execute their various effects (Charmandari et al., 2005; Helmreich et al., 2005). Various internal signals, e.g. central nerve systems (limbic system, brain stem, thalamus, etc.), peripheral hormones, negative feedback loops; and external signals, e.g. the environment, reproductive status of the animal, can have a negative and/or positive effect on the cascade. Glucocorticoids can also bind to mineralocorticoid receptors (MR), present in the HPA regulatory centres of the brain (Norman and Henry, 2015c; Sheriff et al., 2011).

2.4.5 Stress and stressors

Stress is a vague term, often used to describe both the stressor, the response as well as the pathological consequences. The ambiguity of the word stress caused the emergence of the term "allostasis". Similar to the stress response, allostasis is defined by McEwen and Wingfield (2003) as: "the process of maintaining stability (homeostasis) through change in both environmental stimuli and physiological mechanisms". The allostasis model uses 'allostasis load': a term to describe the energy increase needed to deal with stressors and maintain homeostasis (Romero et al., 2009). To cope with (environmental) changes and maintain homeostasis, animals have developed several physiological adaptations, such as (1) increased cognition, alertness, and temperature, (2) suppression of appetite and reproduction, (3) shunting of oxygen and nutrients to the central nervous system (CNS) and the strained body parts, (4) increase in cardiovascular tone, respiration rate and metabolism. These adaptations facilitate an animal's escape from life threatening situations.

When no stressor is present, AVP and CRH will be secreted in a circadian, pulsatile rhythm. Amplitude changes can be seen early in the morning. When acute stress occurs, the PVN gets stimulated leading to an increase in the excretion of CRH, AVP and subsequently ACTH, eventually increasing the production of glucocorticoids, above basal levels (Charmandari et al., 2005). In healthy vertebrates, this pathway and the subsequent GC increase takes three to five minutes and highest levels are reached after 15-30 minutes, after which glucocorticoids return to the base levels after 60-90 minutes (De Kloet et al., 2005).

Stress is dynamic and depending on the nature, severity and duration of the stressor, the magnitude of response can vary (Sze and Brunton, 2020). In normal conditions, the hippocampus inhibits the HPA axis. When GC levels rise, they have a negative effect on CRH and ACTH production, creating a negative feedback loop that can terminate a stress response and regulate the normal circadian rhythm of GC. This feedback system operates best in acute stress situations and is weaker when a stressor is chronic, resulting in a longer stress state. When a chronic stressor is present and glucocorticoid levels remain high for long periods of time, this can be harmful to the animals' fitness and cause numerous of pathologies. Prolonged activation of the stress axis can lead to suppression of growth hormone, gastrointestinal function and immune response (Charmandari et al., 2005; Sheriff et al., 2011). Chronic stress also negatively influences multiple components of the reproductive axis, thus supressing reproduction. CRH inhibits GnRH-, LH- & FSH-producing cells and in consequence gonadal steroid production (Charmandari et al., 2005). Longitudinal measurement of glucocorticoid levels can give insight into an animal's well-being and aid our understanding of their conservation needs, health status and management issues.

As shown in figure 6, there are many interactions between the HPA and HPG axis. Even though basal glucocorticoids can aide reproductive function, stress-induced levels will supress it. Regulation of reproductive steroids, by glucocorticoids, can happen at both the central and peripheral side of the axis (Whirledge and Cidlowski, 2017).

Figure 6: Schematic representation of the interactions between the stress system and the hypothalamic-pituitary-gonadal axis. (from: Charmandari et al., 2005)

CRH (Corticotropin releasing hormone), ACTH (*Adrenocorticotropin hormone*), LH(*luteinizing hormone*, FSH (*follicle-stimulating hormone*), E$_2$ (oestradiol), T (testosterone), LC/NE (*locus coeruleus-norepinephrine*), NE (norepinephrine)

2.5 Non-invasive wildlife endocrinology
2.5.1 Introduction

Hormone analyses may provide extensive knowledge about the basic physiological functioning of an animal. Through the measurement of hormones, more precisely reproductive and adrenocortical steroids, a better understanding of the reproduction, health, and stress sensitivity of an animal can be generated (Ganswindt et al., 2012). Knowledge of the endocrine metabolism of a pangolin is vital for its conservation and management. With an understanding of the oestrus cycle, length of gestation, pregnancy rate and age of onset of puberty as well as senescence, rehabilitators and conservationists can predict population growth or decline, and manage accordingly. This information can thus be used as a tool in their conservation management (Schwarzenberger, 2007; Silva et al., 2017; Volpato, 1999; Wolf et al., 2019).

Blood samples have been traditionally used for steroid analysis, providing an accurate and real time reflection of the hormonal status. Blood samples also often allow the use of commercially available assays, based on monoclonal antibodies directed against unmetabolized steroids. Sample extraction steps are often limited, which allows a faster analysis compared to more complicated matrices such as faeces and urine. However, due to the impracticality of sampling wild animals (restraint is often necessary), the risk to damage vascular beds (repeated sampling), infection and the acute stress response evoked by sampling (potentially also altering glucocorticoid levels), endocrine monitoring of wild animals happens preferably through non-invasive sampling methods. If evaluation of the total
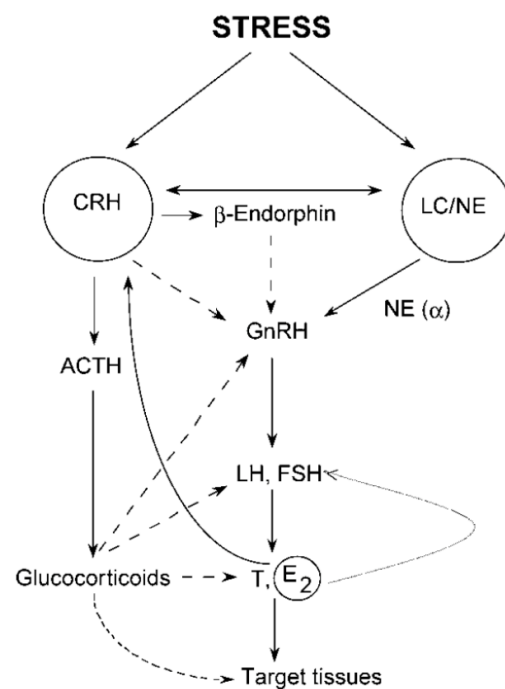
glucocorticoid fraction is needed, blood samples are the only option, since other matrices only show the unbound fraction (Heistermann, 2010; Kleiman et al., 2010; Peter et al., 2018; Silva et al., 2017; Wolf et al., 2019).

As a result, over the past 25 years, non-invasive hormone monitoring has gained popularity. Presently, hormones can be measured in several matrices other than blood e.g. urine, faeces, saliva, and hair. The choice which matrix to be used, is dependent on a range of factors, including the type of information needed, the available analytical techniques, species differences in hormone metabolism and excretion, and the practicality of sample collection (Ganswindt et al., 2012; Heistermann, 2010; Silva et al., 2017).

2.5.2 Minimally invasive matrices

Hormones enter the **saliva** trough passive diffusion. Therefore, they are not modified and identical to those found in blood. This allows the use of EIA assays designed against unmetabolized steroids. Hormonal changes are detectable 20-30 minutes after systemic release (Sheriff et al., 2011). This short lag-time makes saliva useful for detecting short-term hormonal fluctuations, reflecting the changes in the blood. Certain hormones, such as cortisol and testosterone show a variation in the secreted concentrations over the day e.g. the release follows a diurnal pattern. These diurnal fluctuations, seen in the blood, can thus also be seen in the saliva, making samples sometimes difficult to interpret (Heistermann, 2010; Hodges et al., 2010; Kleiman et al., 2010; Silva et al., 2017) Saliva samples have been demonstrated to be successful for progesterone monitoring in the African Elephant (*Loxodonta Africana*) (Illera et al., 2014). However, the need for relative close contact or trained animals for collection, makes it less suitable for use in wildlife (Wolf et al., 2019). In untrained animals, restraint and sedation will still be necessary, causing stress with negative impact on the animals' physiology (Heistermann, 2010). In general, analysis of saliva samples has been more successful for glucocorticoid monitoring compared to reproductive monitoring (Hodges et al., 2010). Glucocorticoids are stable in saliva and are best kept frozen at -20°C (Sheriff et al., 2011).

Hair, another non-invasive matrix, is also less intensively used. Hair is often collected through clipping, for which restraint of the animal is required, making the sampling not completely non-invasive. Although the available hormones are structurally similar to the ones in the blood, limited information can be obtained. Some studies using hair progesterone levels for determining reproductive function in animals, found a weak correlation between hair progesterone concentrations and ovarian activity (Tallo-Parra et al., 2018). At present, it is used mostly for monitoring chronic stress through hair glucocorticoid levels (Peter et al., 2018; Silva et al., 2017). The extraction of hair samples is more complicated compared to other non-invasive matrices. For example, to avoid contamination from sweat and saliva, samples should be washed with methanol or another organic solvent prior to analysis.

Urine, sample extraction is fairly easy. However, to compensate for differences in urine concentration, creatinine levels or urinary specific gravity should be determined (Heistermann, 2010; Hodges et al., 2010; Kleiman et al., 2010; Volpato, 1999). This matrix can contain various classes of hormones, e.g. steroids, proteo-hormones, glycoprotein hormones (chorionic gonadotropin), peptide hormones, catecholamines, making it a suitable matrix for many studies (Ganswindt et al., 2002; Heistermann, 2010; Roth, 2006). Lag times usually range from 2-14 hours (Hodges et al., 2010). The biggest disadvantage is the collection. Obtaining urinary samples happens most easily through aspirating from a non-absorbable surface. Unfortunately, under field condition, the ideal substrate for aspirating is rarely present.

Faecal samples are most practical to obtain non-invasively, making them ideal for longitudinal studies in the wild. Retrieval of the samples is possible through tracking or even dog scat detection (Kleiman et al., 2010; Schwarzenberger, 2007; Wasser et al., 2004). However, there are quite a few disadvantages linked to the processing and analysis of the samples. Faecal samples must be stored fresh at -20°C immediately after sampling, or if not possible, be lyophilized or immediately extracted, otherwise bacterial degradation and steroid concentration changes may occur. Storage in ethanol is also possible,

but not a long term solution, because significant steroid concentration alterations can develop (Hodges et al., 2010; Sheriff et al., 2011). The samples must also be homogenized (because of the uneven distribution) and need to undergo several extraction steps before analysis (Heistermann, 2010; Kleiman et al., 2010; Peter et al., 2018; Schwarzenberger et al., 1996; Silva et al., 2017). To compensate for the total amount of a variable faeces volume, hormone levels are often expressed per unit dry or wet weight (Heistermann, 2010). Faecal samples have a considerable lag time (the delay between hormone secretion and excretion), which can vary from 6-48 hours depending on the species, diet, stress level and overall health of the animal (Hodges et al., 2010). This lag time can be beneficial when measuring glucocorticoids. The absence of stress induced fluctuations and circadian influences allows the measurement of basal free GC values (Sheriff et al., 2011).

Because of variation in steroid metabolism, different mammalian species generate different metabolites, even in closely related species, resulting in species specific steroid metabolites. Besides the different steroid metabolites produced, excretion pathways also differ between animals and between steroids within the same species. It has been reported that felids excrete steroid metabolites almost exclusively in faeces (Kinoshita et al., 2011). Whereas, the bottlenose dolphin (*Turciops truncates*) excretes reproduction hormones predominantly in urine (Romano et al., 2010).
Ultimately, the most important factor when choosing a non-invasive matrix, is the feasibility of obtaining a valid sample, and the metabolic excretion pathway of the species in question (Schwarzenberger, 2007).

Radiometabolism studies are an elegant way to not only map the excretion pathways, but also to identify compound-specific metabolites and to measure time lag between blood and excretion into non-invasive matrices. In these studies, a radiolabelled steroid hormone gets injected into the animal and the subsequent excreta are collected and analysed, through the use of liquid chromatography separation (Kersey and Dehnhard, 2014; Palme, 2019; Pribbenow et al., 2014; Schwarzenberger et al., 1996).

2.5.3 Radio immunoassay

Radioimmunoassay (RIA) and enzyme-immunoassay (EIA) are the two most commonly used analytical techniques, historically applied in non-invasive wildlife monitoring. They are based on the biochemical principle of antigen binding to its specific antibody with the aim to measure small quantities of antigens (hormones, proteins) or antibody (Brown et al., 2004; Gan and Patel, 2013)

Radioimmunoassay's use a predefined amount of antibody and a radio-actively labelled antigen 'tracer'. After separation of the bound and free tracer fraction, radioactivity can be measured and used to calculate the steroid concentration. RIA's are sensitive, specific and are relatively easy to conduct, however they are not without flaw. The main disadvantages of RIA are the radio-isotopes which need to be properly disposed of, the costly equipment, need for a license and the health hazards (Gan and Patel, 2013; Schwarzenberger, 2007; Van Weemen et al., 1979; Wudy et al., 2018).

2.5.4 Enzyme immunoassay

Enzyme immunoassay's (EIA) are derived from the RIA principle, replacing the radioactive tracer with an enzyme. EIA uses tracer antigens linked with enzymes who convert an added substrate into a colorimetric signal (Gan and Patel, 2013; Midhun et al., 2021). Comparing the colour intensity of samples to a previously established serial dilution standard curve, allows the qualitative determination of target hormones in the sample (Alhajj and Farhana, 2022). EIAs have been gaining popularity since they often nearly attain the same sensitivity level as RIA (Van Weemen et al., 1979). Due to the enzymatic process, there is a significant amplification of the signal, increasing the sensitivity (Ngel Gonzá Lez-Martínez et al., 2018). Additionally, they can be performed with less expensive equipment and without the need for radioactive substances. Another major advantage of EIA is the possibility to use group specific antibodies, which allows analysis of metabolites with a common chemical structure (Ballester Cruelles, 1998; Gan and Patel, 2013; Sheriff et al., 2011).

Because of these mentioned advantages, and its wide usage in non-invasive endocrinology, it was chosen to focus on EIA as the preferred analytical technique for this master dissertation. The applicability of this technique in non-invasive wildlife endocrinology will therefore be described into more detail below.

2.5.4.1   General principles

Enzyme immuno-assays are essentially analytical detection methods, that use specific antibody binding to detect analytes (antigens, antibodies, biomolecules) in complex matrices (serum, plasma, urine, saliva, faeces). The antibody-analyte binding is linked with an enzyme, giving a colorimetric change that enables the methodical quantification of the analyte. Of EIA's, the immunosorbent assay (ELISA) is the most commonly used. ELISA's use a solid phase that allows the separation of bound and free fraction, making them more complex but also more precise and sensitive (Van Weemen et al., 1979). ELISA has been employed for steroid monitoring in various species, e.g., white rhinoceros *(Ceratotherium simum simum)* (van der Goot et al., 2015), the African elephant *(Loxodonta Africana)*(Ganswindt et al., 2002)*,* the giant anteater *(Myrmecophaga tridactyla)* and several *Felidae* species (Adachi et al., 2010; Kinoshita et al., 2011; Pribbenow et al., 2014; Umapathy et al., 2013a). In the direct ELISA, the simplest approach, a buffered analyte (antigen, hormone, antibody) is fixated, usually on a 96well polystyrene microplate. Next, any unbound sites are blocked with Bovine Serum Albumin (BSA) or any other non-reactive protein. After this, a specific primary antibody (enzyme conjugated) is added, which will detect the analyte of interest's binding site. Substrate is added and converted by the enzyme. The wells are read, and colour changes detected. Between these steps the plates are washed a number of times with a buffer, removing any unbound material (Alhajj and Farhana, 2022). Common enzymes used are alkaline phosphatase (AP), glucose oxidase (GO) and horse radish peroxidase (HRP) (Gan and Patel, 2013).

The type of antibody used, can be classified into one of two groups. **Monoclonal antibodies** have a great sensitivity and are less susceptible to disturbing factors. However, their production is expensive and time consuming and they can only be used for macromolecules (Ngel Gonzá Lez-Martínez et al., 2018; Porstmann and Kiessig, 1992). **Polyclonal antibodies** on the other hand can bind a mixture of epitopes, allowing small changes of the antigen, resulting in high affinity and high sensitivity. Less ideally, this makes them cross react more frequently (Ngel Gonzá Lez-Martínez et al., 2018). **Group specific antibodies**, cross react with structurally related metabolites. As mentioned earlier this can be an advantage, if the exact sample target cannot be determined. However, when possible cross reactions with structurally related but physiologically different metabolites occur, this results in false positive results. Careful validation is needed when using these antibodies in assays (Hodges et al., 2010; Sheriff et al., 2011).

The direct ELISA is not very sensitive and can only be used with macromolecules. As a result, other types of EIA have been created, e.g. competitive, indirect, indirect competitive (inhibiting) and sandwich EIA, shown in figure 7. Choice of method will depend on the study objection, type of sample, concentration of the analyte and chosen type of antibody. The **Indirect ELISA** uses 2 different antibody types. The primary antibody binds to the antigen, and a secondary enzyme conjugated antibody binds to the primary one. The use of an extra antibody raises the assays' sensitivity level, but also increases risk of cross-reactivity (Alhajj and Farhana, 2022; Gan and Patel, 2013).
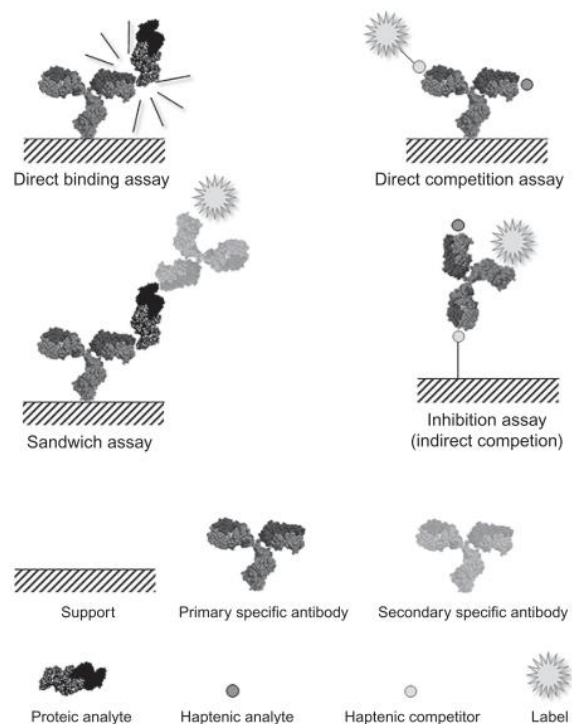

Figure 7: Different ELISA types, simplified (from: Ngel Gonzá Lez-Martinez et al., 2018)

When a sample of mixed antigens is used, **a sandwich ELISA** could be beneficial. This ELISA uses a separate purified and specific capture antibody and detection antibody, "sandwiching" the antigen between both. With this both sensitivity and specificity increases. Sandwich ELISA's have the best sensitivity of all, thus are ideal for low concentration substances, but can only be used for macromolecular targets who have at least two different epitopes (Alhajj and Farhana, 2022; Ngel Gonzá Lez-Martínez et al., 2018).

Competitive ELISA's are mainly used if high sensitivity is needed in a complex mixture but the antigen is small and/or only has one epitope. Specificity is lower, however. In this type of assay, the sample analyte competes with a labelled analogous molecule (tracer) for binding on a limited number of antibodies. Cumulative competition occurs and the antigen present in higher concentration will bind to more antibodies. More antigen in the sample, means less tracer binding and consequently less chromogenic response (Alhajj and Farhana, 2022; Ballester Cruelles, 1998; Gan and Patel, 2013; Wudy et al., 2018). In the **indirect competition assay**, the competiting conjugate is bound to a specific antibody on the surface plate. Depending on the concentration of antigen present in the sample, more or less conjugate will bind to the specific antibody. Dependant on the final bound amount, the strength of the fluorescent signal will vary (Ngel Gonzá Lez-Martínez et al., 2018).

2.5.4.2 Analytical validation

Validation of a method ensures that obtained results are qualitative, accurate and reliable in the context of the methods intended use. Especially considering the excretion variability between species, it is crucial to validate each assay per species-matrix combination of interest. Laboratories will use slightly different criteria, but in general validation includes four main requirements: Sensitivity, precision, accuracy and specificity (Hodges et al., 2010; Minic and Zivkovic, 2020; Palme, 2019).

Optimizing the assay, making it as functional and effective as possible, will benefit results in the end. Each separate step, of which choosing the assay type (direct, indirect, sandwich) is the most important one, should be optimised in advance of the assay validation. Numerous factors should be tested, including: antigen coating, saturation-blocking, sample preparation, choice of antibody, enzyme conjugate selection, signal detection (Minic and Zivkovic, 2020). **Robustness** which entails the ability of the method to remain unaffected by small unintentional errors in method implementation, is established during optimization (Andreasson et al., 2015; Minic and Zivkovic, 2020; Wallwitz et al., 2019).

Depending on several factors, e.g., selected assay type, substrate and enzyme choice, the **sensitivity** level of ELISA's range from femtomoles to µmoles per tube (Van Weemen et al., 1979). The assay's sensitivity depends on its ability to detect a specified concentration of analyte and is often defined by a detection limit. The limit of detection (LD), limits of quantification and linearity can affirm the sensitivity. Limit of detection (LD), is the lowest concentration that can be determined and differentiated from the background noise, calculated using blank test series. Similar is the lower limit of quantification (LLOQ), which also demonstrates the lowest detectable concentration of analyte, but with acceptable levels of precision and accuracy (Andreasson et al., 2015). Linearity shows the assay's ability to measure diluted samples. When linearity is present, the measured results are directly proportional to the concentration of analyte in the sample. Therefore, allowing excessive concentrations of analyte to be measured and calculated after dilution. Presence of linearity can be demonstrated through linearity of dilution tests. Recovery percentage after dilution, should fall between 80%-120% of the expected values in order to achieve linearity. From the linearity test, the upper and lower quantification limits (ULOQ and LLOQ respectively) can be determined, with this establishing the range of linearity (Minic and Zivkovic, 2020). A rare but important pitfall of enzyme immunoassays is the **hook effect**, supressing signals, when extreme high concentrations of analyte (above the ULOQ) are present in the sample. The resulting saturation leads to falsely decreased levels. Reanalysis after dilution is in this case necessary (Wudy et al., 2018; Andreasson et al., 2015).

Precision describes the reproducibility of a measurement, when tested on the same sample in the same conditions. When successive measurements show close results, high precision is present. It is

represented by the coefficient of variation (CV) or relative standard deviation (RSD), which is similar to the standard deviation but derived from the measured mean value and expressed in percentages. Precision includes the standard deviation (SD), CV and confidence interval (Wallwitz et al., 2019).
It can be attained at different levels: 1. Intra assay precision or repeatability, ideally 10% or less, describes the precision between repeated measurements of the exact same sample, between wells within the same assay. When precision is high, a repeated measurement should give identical or at least comparable results. 2. Intra assay CV % is the average CV%, calculated from the individual CV's% of each sample (Minic and Zivkovic, 2020). 3. Intermediate precision compares assay's executed on different plates and different days, but performed in the same laboratory. Instead of intermediate precision inter assay precision is commonly used, however inter assay precision or reproducibility, indicates a comparison of values between laboratories, demonstrating standardization possibilies. Standardisation is needed, in order to reliably compare results obtained from different laboratories (Minic and Zivkovic, 2020). High degree of imprecision, might be due to random errors (Elmlinger, 2011).

Precision, although similar, differs from **accuracy**. In contrast to precision, accuracy defines the deviation from the nominal values and not from the mean value. High accuracy, results in a good correlation between the obtained assay concentrations and the reference values. The difference between these is defined as the 'the absolute error'. Derived from this is the relative error, expressed in percentages. Level of accuracy should be determined for the ULOQ, LLOQ and midrange (Elmlinger, 2011; Minic and Zivkovic, 2020; Sheriff et al., 2011). Accuracy is evaluated through spike-recovery- and linearity assays. Spike and recovery compares spiked sample with blank sample concentrations. Alternatively, quality control tests, in which the analyte concentrations are known, can also validate assay protocols. In ideal situations concentrations are the same and recovery is 100%, still deviations of maximal 20% are allowed. Significant differences indicate a systematic error and interfering factors in the sample matrix. Alternative diluents or ratio's might be required to solve this issue (Andreasson et al., 2015; Minic and Zivkovic, 2020). Relative accuracy of a diluted sample is defined by **parallelism**. In contrast to dilution linearity, parallelism tests do not spike but use high endogenous analyte concentrated samples, below the ULOQ. This way, sample matrix effects are evaluated without the dilution effects seen in linearity tests (Andreasson et al., 2015).

To minimize false positive results, which results in an overestimation of the analyte, high **specificity** is required. A specific assay protocol is able to differentiate target analytes from other chemically related components present in the sample matrix. Therefore, evidently cross reactivity needs to be limited. This can be evaluated by adding increasing concentrations of related molecules into the sample matrix, and assessing at which level binding occurs (Elmlinger, 2011; Minic and Zivkovic, 2020; Sheriff et al., 2011). For reproductive steroids where cross reactivity is tested separately for each oestrogen, Van Weemen et al. (1979) found that in the presence of oestrogen cocktails, there might be an overestimation, possibly because of a concerted action of the steroids.

2.5.4.3 Biological validation

In order to demonstrate the significance of hormonal fluctuations measured, biological validation is necessary. This can be done by comparing hormone concentrations between matched urinary and faecal samples., between matched samples (i.e. faecal vs urinary oestrogen).

Another way is to link the measured hormonal concentration, to a predicted change or difference in hormones, e.g., observed external signs of oestrus, sexual maturity, or in the case of GC biological validation, linking multiple faecal tests to a stressful event. A commonly seen pregnancy pattern, concurring with pregnancy and/or parturition, can also validate the obtained concentrations (Kersey and Dehnhard, 2014; Brown et al., 2004).

A more invasive way of validating, includes the administration of a stimulating drug or a "hormonal challenge", e.g. injecting GnRH to study the pituitary hormones (FSH, LH). Hormonal challenges will also elucidate any excretory lag time present. Blood samples are often used as part of the validation, by comparing blood with the excreted hormone levels (Hodges et al., 2010; Kersey and Dehnhard, 2014; Brown et al., 2004).

2.5.5 Endocrinological monitoring pangolin

Not much is known about the reproductive biology of pangolins, and even less is known about the major reproductive steroid metabolites present in their faecal material. To date, only three studies on reproductive metabolites in pangolins have been performed. Chin et al. (2012), proved that progesterone radioimmunoassay on serum, is an effective method to assess the reproductive status of the Chinese pangolin. They successfully determined pregnancy and estimated the gestation period. Alternatively, through the use of non-invasive faecal matter and electrospray ionization mass spectrometry (ESI-MS/MS), Arora et al. (2020), identified oestradiol-17β and allopregnanolone-5a as the principal metabolites in Chinese pangolins. They also found that adult females possess higher estrogen metabolite concentrations, then sub adults. For the Temminck's pangolin, Blecher et al. (2021) successfully measured progestogen metabolites from scales, through the use of an Enzyme immuno-assay.

Considering the limited information on the Pholidota order, a carfull comparison with the most closely related sister taxon, the order of the Carnivora could be made. The Carnivora order, has been studied more intensely. For most big cats (Asiatic lion (*Panthera leo*), tiger (*Panthera tigris*), leopard (*Panthera pardus*), it is found that oestradiol-17β and estrone are the predominating oestrogen metabolites and 5β- and 5α- reduced pregnanes, the major progesterone metabolites in faecal samples (Umapathy et al., 2013b). It has also been shown that oestrogens in carnivores are predominantly excreted into the faeces, making the determination of ovulation via preovulatory oestrogen surge detection possible (Schwarzenberger et al., 1996). Extrapolation from the carnivora order, is possible, but should be done cautiously. Species variation should be considered, and validation of the protocol is necessary.

3. Problem statement and research objective

Literature review shows a knowledge gap and a lack of data on the reproductive parameters of all pangolin species. More specifically: the oestrus cycle and duration of its different stages , gestation time, gonad activity and the absence or presence of seasonality. Some research on this has been done for the Chinese pangolin (Chin et al., 2012; Zhang et al., 2016, 2015), unfortunately this is limited, and its results vary considerably, thus giving rise to some discussion and uncertainty. Furthermore, little to no research has been conducted for the Temminck's pangolin. Obtaining this information can, as mentioned earlier, be used in pregnancy determination, benefitting conservation aids.

In order to bridge this gap, this study aims to map the oestrous cycle length and oestrus cycle fases (follicular and luteal) of the female Temminck's pangolin, including the comparison of the luteal phase between a pregnant and nonpregnant animal. Because this study involves wild and threatened animals, non-invasive long-term monitoring, using faecal metabolites will be most appropriate. EIA's have been previously shown successful in determining hormone concentrations in other wildlife. Whilst numerous endocrine studies using EIA-techniques have been performed, only one has been conducted on the Temminck's pangolin before. Considering the variability in metabolism and excretion of steroids, extrapolation between animals and even species is not advisable. Therefore, during this research, we attend to establish and properly validate a reliable ELISA protocol, for the specific use of non-invasive endocrine monitoring of the Temminck's pangolin. Hereby focussing on determining the major faecal oestradiol and progesterone metabolites, E2 and P4 respectively. Hopefully, the established protocol can then be used for pregnancy diagnosis via faecal analysis in the future.

A big challenge for every wild animal, but especially in endangered species, is the compilation of a valid sample set. For this study, longitudinal samples and proper storage at -20°C are needed. This is not always feasible in wildlife settings, therefore rehabilitation animals seem most ideal. The samples will need to be transported and analysed at a suitable laboratory.

4. Materials and methods

4.1 Study population

This research will be done in cooperation with the Johannesburg Wildlife Veterinary Hospital, a non-profit Wildlife Veterinary Hospital and Rehabilitation facility, under supervision of Dr. Karen Lourens, the co-founder and veterinary surgeon. Their resident Temminck's pangolins, are poached or injured animals who are treated and rehabilitated at the hospital, until their release back into the wild. Duration of their stay varies, and depends on their health status, usually this is at least two to three weeks. The pangolins are housed individually, in wooden boxes, simulating a burrow. During their daily, five-hour long walk, they are able to forage freely and get their natural diet of ants and termites. These walks also allow them to engage in their natural behaviour and reduces stress.

4.2 Sample collection

In order to obtain enough information and have representative results; a minimum of six individuals will be sampled, with three pregnant and three nonpregnant animals. Ideally each pangolin is sampled during longer periods of time, making it more likely that entire cycles are sampled. However, considering sample collection will only happen during the hospitalisation and the rehabilitation period, logically, sampling will be stopped once an animal is released into the wild. In order to still collect enough, sampling will start in April 2021 and continue until analysis in January 2022.
To limit the disturbance, the voided faecal samples will be collected during staffs' regular rounds, usually in the afternoon, right before they go on their daily walk. Following a defined sample schedule, nonpregnant animals will be sampled daily and confirmed pregnant animals get sampled two to three times a week. An approximate amount of 10gr will be sampled each time, and immediately stored and frozen (-20°C) in a marked plastic bag, until further processing. Confirmation of pregnancy in suspected 'pregnant' animals will happen via ultrasound.
For each pangolin the following additional information as shown in table 1, will be noted.

Table 1: Additional information, noted during sample collection.

Pangolin ID	Date and time of collection	Pregnant / nonpregnant / unsure	Approximate freshness
Day 1 of sampling			
Day 2 of sampling			
Day 3 of sampling			
Day 4 of sampling			
Etc.			

4.3 Transportation

Because analysis of the samples is not possible at the site of collection, two options for transport and analysis have been evaluated.
Firstly, shipment of the samples to Berlin, for analysis at the IZW institute was considered. After further research into the mandatory papers and receiving a quote on temperature-controlled shipment costs (€1,128 and €2500), this option was deemed unrealistic and discarded.
The second option is analysis at the Mammal Research Institute, Endocrine Research Laboratory at the University of Pretoria SA, where Prof. Dr. Andre Ganswindt kindly offered to aid in the analysis. Even though the analysis is happening locally, in order to conduct the research and transport the samples in South Africa, a section 20 permit will be needed. Transportation of the samples will happen cooled and controlled, in accordance to the *national road traffic act, 1996* (SA).

4.4 Permits

<u>Section 20 permit</u>

The *Animal Diseases Act 35, 1984* s.20 (SA). States that "Any research falling under section 20 of this act, requires a section 20 permit, granted by the National Director of Animal Health". The purpose of the permit is to prevent possible disease transmission caused by animal products and or materials.

Applying for a section 20 permit is a lengthy process, requiring numerous signed, official papers.
The application form, provided by the South-African Agriculture, Forestry and Fisheries department can be found online. Usually the full application process takes about three months. In the form, the research title, starting date and methodology will need to be provided; as well as, information on the 'animal products' and its intended use. With this, clarifying the scope of the study.

The disposal methods and facilities used need to be stated and accompanied with the biohazard and waste certificates of the laboratory. In addition to this, several other official paperwork need to be attached with the form.
Firstly, because the study involves critically endangered species, a copy of the JWVH TOPS (Threatened or Protected Species) permit has to be provided. Additionally, a signed, official letter from the research facility (Dr. Nicole Hagenah-Schrader), JWVH's responsible veterinarian (Dr.Karin Lourens) and the state veterinarian (Dr. AMM Grobler) responsible for the abattoir will be needed.
In his letter, Dr. Grobbler, state veterinarian of the regional abattoir, will have to clarify whether the sample area is under any official quarantine and state his awareness of the study purpose and its non-invasive character. Dr. Karin Lourens will have to grant her authorisation of the study, using the animals under her care. Finally, Dr. Nicole Hagenah-Schrader has to state how all samples will be stored, processed and disposed of in relation to the research objectives.
All of these letters will need to be on the official letterhead, signed and converted into PDF format.
Lastly, for every research, including this one, an ethics permit will be required.
Finally, the documents will need to get signed by the supervisor of the research project (Prof. Dr. Andre Ganswindt), the person in charge of the main laboratory facilities (Dr. Nicole Hagenah-Schrader, the Endocrine Research Laboratory Manager), and the person responsible for research (myself, Ilke Van Dooren).

After obtaining all this information, letters and permits, I will apply for the section permit, via email to the Control Veterinary Technologist, Marna Laing.

<u>Ethics permit</u>

Because this research does not involve any disturbance or actual contact with living animals, for the University of Ghent, ethical approval will not be necessary. However, with the application of the section 20 permit, an ethics permit needs to be added.
Ethics permits for research purposes in master dissertations and PHD's are usually requested through the ethics board of the student's university.

4.5 Sample analysis

Initially, analysis of the samples would have happened in January 2022. During this month, I had taken time off from clinic to assist in the lab, where needed. Being present whilst the analyses are performed would greatly expand my knowledge and skills in EIA-techniques and help me understand the reasoning behind the evaluation steps.

Unfortunately, sample analysis couldn't take place yet, due to unexpected challenges during sample collection, permit requirement and Covid restrictions, which will be further elaborated on in the

discussion. Sample collection will continue during the following months and analysis is planned for later this year.

5. Results

Unfortunately, the analysis planned for January 2022, had to be delayed, and has not been performed yet. As a consequence, there are no data results thus far.

<u>Samples</u>

Due to an unforeseen power outage at the JWVH, a lot of the samples were lost. The previously set amount of longitudinal sample sets of 6 pangolins could therefore not be achieved yet. The current number of samples is shown in table 2 below. They are stored at -20°C in the freezer of the rehabilitation centre of JWVH, until enough samples are collected. At the moment, there are two pregnant pangolins still under treatment at the JWVH, they are both being sampled. After a sufficient number of samples is secured, they can be prepared and transported for analysis.

Table 2: Sample's acquired from JWVH until now.

Pangolin ID	Pregnant / nonpregnant / unsure	Number of samples already collected
Pangolin N°1	Pregnant	8
Pangolin N°2	Pregnant	10
Pangolin N°3	Pregnant	4
Pangolin N°4	Pregnant	1
Pangolin N°5	Pregnant	0

<u>Section 20 permit</u>

The section 20 permit, needed for the possession and translocation of S.*temminckii's* faecal samples was applied for and received from the department of Agriculture, Land Reform and Rural Development (DALRRD) of the Republic of South Africa, on March 23[rd,], 2022. In total the whole process, from information gathering to acquiring took 4 months (Started on the 11th of November). The permit is valid until December 31st, 2023, which should be sufficient time for the transport and analysis.

<u>Ethics approval</u>

Obtaining an ethics permit through the Ethics Committee of Ghent University was not possible in my case. Considering the analysis will take at the University of Pretoria and only studies performed at the laboratory of Ghent are eligible for ethics approval.
Similarly, an ethics approval could not be granted through the Faculty of Health Sciences Research Ethics Committee of the University of Pretoria, because only students registered at their faculty can qualify for this.
Eventually this issue was resolved thanks to Dr. Jella Wauters , and the ethics permit was granted by the Internal Committee for Ethics and Animal Welfare of the IZW (Leibniz-Institut für Zoo- und Wildtierforschung).

6. Discussion and conclusion

The main objective of this master dissertation was to determine the main faecal oestrogen and progesterone metabolites of the female Temminck's pangolin, through the use of enzyme immunoassays. As another objective, it was intended to use these results to further our understanding of their oestrus cycle. Thirdly, the established and validated EIA-protocol in combination with the difference in oestrogen and progesterone concentrations between pregnant and nonpregnant animals, will hopefully make non-invasive faecal pregnancy detection possible in the future. Lastly, additional knowledge on other aspects of their reproduction, e.g., onset of puberty, seasonality, spontaneous versus induced ovulators, etc. might be discovered.

In this master dissertation a short literature overview has been given, illustrating the need for this research. Different Elisa-types are discussed, as well as the extensive validation needed when developing a still unused EIA protocol for a new species-matrix combination.

Unfortunately, due to multiple difficulties, the analysis could not be performed during my master dissertations' timeframe. Consequently, there are no results yet regarding oestrogen and progesterone concentrations, which inevitably prevents the realisation of any of the objectives.
A confluence of obstacles has prevented the analysis from happening in January.
The first problem was the **section 20 permit**. Because our initial plan was exporting the samples to Berlin, I spent the majority of the prepatory fase (the year before the dissertation) figuring out the necessary documents and contacting transport companies for shipment. After the received quota were deemed too high, the better alternative of local analysis with the help of Prof. Dr. Andre Ganswindt was chosen. At this point it was already October 2022. I did not realize a section 20 permit was necessary, partly because of the late trajectory switch and partly because of my lack of knowledge of South African regulations, and this caused a late start in applicating for the permit. Thankfully both Dr. Karin Lourens and Dr. Andre Ganswindt mentioned the need for this permit, because taking into account the close vicinity of the JWVH and the Endocrine Research Laboratory, a need for a permit did not even cross my mind. With the guidance of Dr. Andrea Webster, I managed to obtain all the necessary documents and received the permit on March 23rd 2022. Although procuring the permit generally does take a long time, in my case it even took 4 months. Possible reasons for this are my lack of knowledge in this area and the unfortunate timing (holidays). The ethics permit needed for the section 20 permit was also not easy to obtain. As mentioned in the results, due to an incongruency between the different ethics committees, I did not qualify for an ethics permit at either. Therefore we needed to resort to an alternative, in my case the IZW Internal Committee.
Due to this delay, sample transport and analysis could not happen during the planned month in January.

A second complicating factor was the **Covid-19 pandemic**, which greatly complicated travel between Belgium and South Africa. During the month of January, in which analyses were originally planned, the Covid-19 situation was bad and heavy travel restrictions were implemented. Taking into account both the permit delay and Covid-19 restrictions, analysis was postponed. Later in March with the section 20 permit obtained and the Covid-19 regulations eased, analysis of the samples during March-April seemed plausible. Originally, I was planning on travelling to SA, to help in the analysis and become familiar with their ELISA technique. However, travel restrictions and shift of the timing to March, made travel for me impossible, additionally I could not get any more time off from clinic rotation at the faculty.
As a solution, the analysis will be performed by Prof. Dr. Andre Ganswindt and his laboratory staff. Considering the laboratory's heavy workload and other studies, the analysis will have to be fitted in with the other tasks, and will happen when there is time.
A third big obstacle occurred during preparation of the samples for transport. South Africa often experiences electricity shortages. Because of this ,'load shedding' or power outages to save electricity occur regularly. One of these power outages caused **a freezer malfunction** and this was noticed too late, resulting in all samples decomposing and becoming useless.
All these reasons combined, made sample analysis impossible during the timeframe of the master dissertation.

In retrospect, if I had known about the section 20 permit earlier and hence started its application sooner, and Covid-19 had not happened, or had not been as bad during that time; the samples would still have been viable and analysis had most likely taken place during January, leaving enough time for the analysis, processing and data interpretation.

Taking into account my inability to travel to SA, and learn more about EIA, writing the dissertation parts involving EIA proved to be difficult. I spent a long time, simply trying to understand the mechanism, methodology and validation. In hindsight, following the EIA's in real life and helping the analysis would have aided my understanding of this topic. Missing this opportunity to elaborate my knowledge and practice lab techniques is really unfortunate.

To those planning on doing a similar dissertation I advice to look into all the local regulations and practicalities involved beforehand. It takes up a lot of time that you cannot spent on your actual master dissertation.

Even though a lot of difficulties have occurred, sample analysis will still happen, simply delayed. The section 20 permit stays valid until December 31st 2023, which should be sufficient time to perform the analysis. Even though my master dissertation and my official involvement in this research ends the end of June, I would still like to stay involved in the further communication and practical arrangements. The future plan includes: 1. continued sampling, until a sufficient sample set is reached. Ideally consisting of six female pangolins that have been sampled for a longer period of time (a couple of weeks). Very important for the validation are the samples from nonpregnant animals, currently none of these have been taken yet. 2. Preparing and safely transporting the samples, whilst maintaining their integrity. 3. At the Mammal Research Institute, different oestrogen and progesterone assays will be screened for its potential use and the ELISA regarded most suitable will be validated. Most likely a double-antibody indirect competitive EIA type will be utilised, according to the method described by Ganswindt et al. (2002). When validation parameters are satisfactory the samples will be analysed, subsequently the data can be interpretated and the research objectives hopefully fulfilled.

In conclusion, even though the objectives can not be realized yet, the study will continue with these in mind. Hopefully results will prevail interesting and reliable results, applicable in the future conservation of the Temminck's pangolin. Evidenced by the minimal and inconsistent reproductive information on the pangolin described in literature, more research on reprocution still needs to be conducted.

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