Evaluating the utility of using hair for non-invasive endocrine monitoring in the aardwolf (*Proteles cristatus*)

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The main goal of this study is to determine the utility and the possibility of using hair samples for monitoring endocrine parameters in the aardwolf. The endocrine parameters used in this study contain the following hormones: androgens, progesterones and glucocorticoids. Hair of four different regions (neck, belly, left thigh and tail) of four different aardwolves are used for this study. The hair samples were extracted in ethanol and the final solution was pipetted into an EIA microplate. The concentrations found in the hair were compared with the concentrations obtained from faecal samples of the same aardwolves.

Introduction

There are several reasons why development of non-invasive methods for measurement of hormones is highly desirable for wildlife research. Non-invasive techniques reduce pain and suffering for the animals, because these techniques do not enter the body or puncture the skin. These techniques decrease stress, especially when repeated sampling is needed. Therefore non-invasive methods can also be very useful for long term studies.

In addition, wild living animals are often protected species and it can therefore be difficult to get a license for wildlife research. The 'Animal Use and Care Committee' provides a research permit when the research meets certain requirements; the research is necessary, the number of animals is reduced to a minimum, their well-being and welfare standards are ensured and that unnecessary suffering of the animals is excluded *[Website University Pretoria,2010]*. Thus non-invasive techniques are preferred to invasive techniques. Therefore licensing constraints for non-invasive methods of sampling are less restrictive *[Buchanan-Goldsmith,2004]*.

Meanwhile several non-invasive sampling methods are used for different purposes; faecal, urinary, salivary, sweat, feathers, milk, nails and hair sampling *[Cook et al,2000]*. Each method has different advantages and disadvantages (see also appendix 1).

If one is interested in a long term profile, a disadvantage of using faecal, urinary, salivary, sweat and milk is the requirement of a substantial amount of repeated sampling of the same subjects.

Stress involved research will benefit from being conducted on faecal, sweat, feathers, nails and hair samples, since stress hormone levels of these tissues are insensitive for impact of acute stress when handling the animal *[Davenport et al,2006]*. These non-invasive techniques represent baseline conditions, therefore any observed cortisol change is the result of behavioural, reproductive or social events, not related to the sampling itself *[Romano et al,2010]*.

A research that requires measurement of acute hormone responses will benefit to salivary, urinary sweat, and milk samples. For instance salivary and urinary cortisol are appropriate measures of acute stress *[Kalra et al,2007]*.

Hair samples can be very useful for wildlife research. An advantage of using hair samples is that they do not require specific storage or transport. For instance blood, faeces and salivary samples have to be transported frozen or cold *[Koren et al,2002]*. Hair are unaffected in variations of water content and therefore hair does not have to be frozen until the time of analysis, even if analysis is delayed for days, weeks, or even months *[Koren et al,2002]*.

Sample collecting does not entail serious health hazards since there is no involvement of pain or infection during the sampling *[Koren et al,2002]*. Hair is unaffected by daily fluctuations of hormones and therefore also insensitive for the impact of acute stress caused by handling *[Yang et al,1998]*. This makes hair very useful for long term endocrine profiles, like studies of chronic stress and welfare *[Koren et al,2002]*.

The main disadvantages for hair are the possibility of external contamination and the incomplete hair physiology *[Esteban-Catano,2009]*.

During the embryonic stage of life, a hair follicle develops from an ectodermal bud that grows into the underlying mesenchym. The distal end of this bud forms a bulbous enlargement, the dermal papilla, which forms the primitive hair bud *[Dyce et al,2002]*.

A hair shaft consists of three distinct types of cells: an outer cuticle, which surrounds a central cortex which, in turn, may contain a central medulla. The function of the cuticle is to anchor the hair shaft in the follicle and to protect the interior fibers *[Harkey,1993]*. The cortex exist of long keratinized cells which are formed into long fibers. Between the cells of the cortex are very small air spaces called fusi. In the living portion of the hair root, these small spaces are filled with fluid but as the hair grows and dries out, air replaces the fluid *[Harkey,1993]*. Medullar cells are initially loosely packed, but dehydrate and shrivel up to leave a series of vacuoles along the fiber axis *[Harkey,1993]*.

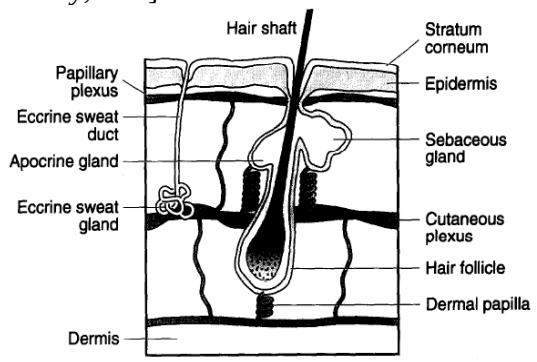

Figure 1. A simplified diagram of a hair follicle, glands and the vascular system *[Harkey,1993].*

Hair follicles are surrounded by two capillary networks, the papillary plexus and the cutaneous plexus (Fig. 1). The cutaneous plexus exist of long parallel vessels connected by cross shunts. These arterial capillaries nourish the growing hair bulb *[Harkey,1993]*.

The nourishing capillaries allow blood borne substances, like circulating hormone molecules, to diffuse into the cells of the hair follicle and subsequently become deposited in the hair shaft. Such diffusion is amplified by high lipid solubility and low protein binding, suggesting preferential deposition of unbound molecules *[Cone,1996; Koren et al,2006]*. Therefore the steroid concentrations measured in hair should reflect the free/ unbound fraction of steroid present in the circulation *[Davenport et al,2006.]*

Hair reflect the hormone levels in the blood circulation and therefore hair assays are used for a variety of purposes: tracing pollutants, drugs, anabolic steroids, determining sex steroids and glucocorticoids *[Koren et al,2002; Yang et al,1998]*. For instance, estrogens, progesterone, testosterone levels measured in healthy human hair correlate with the levels measured in their serum *[Yang et al,1998]*.

The main goal of this study is to determine the utility and the possibility of using hair samples for monitoring endocrine parameters in the aardwolf. The endocrine parameters used in this study contain the following hormones; androgens, progesterones and glucocorticoids. These hormones will give more information about the various aspects of the endocrine metabolism of this species.

Materials

Study site

The study was conducted between November 2009 and March 2010 at Benfontein, an 11.000 hectare game farm near Kimberley, South Africa. The farm is located in a transitional zone where dry Karoo, grassland and Kalahari savannah meet *[Stenkewitz et al,2010]*.

Study Animals

Figure 2. Study animal BWM09008, male aardwolf.

Four radio collared aardwolves *(Proteles cristatus)* were studied (two males and two females). The aardwolf is limited to Africa and occurs in two discrete populations. The southern population ranges over most of southern Africa, extending just into southern Angola, Zambia and Mozambique *[Koehler-Richardson,1990]*. The northern extends from central Tanzania to north-eastern Uganda and Somalia, then narrowly along the coast of Ethiopia and Sudan to the extreme south coast of Egypt *[Koehler-Richardson,1990]*. The research animals belong to this southern population.

The aardwolf is a solitary, nocturnal, medium sized (7-10kg) mammal. Aardwolves feed primarily on termites. Like the brown and striped hyenas, aardwolves have large pointed ears and a long erectile mane extending from behind the head down the middle of the back to the tip of the tail *[Koehler-Richardson,1990]*. Most of the body coat consisted of dense, soft and crinkled under fur interspersed with coarser guard hair. Hair of dorsal crest are coarse and long, being about 70mm on the back of the head, increasing to 200mm on the shoulders, and decreasing to about 160mm on the tail. These hair have broad white bases, then alternating black and white annulations, terminating in black tips *[Wemmer-Wilson,1983]*.

Methods

Sampling

The radio collared (Sirtrack®, Havelock North, approx. weight 60g/ collar) aardwolves are traced by using VHF transmitters.

Figure 3. Collecting hair samples from sedated aardwolf.

The aardwolves were captured by darting the aardwolves with 3.6 milligram ketamine hydrochloride (Anaket-V®) per kilogram bodyweight and 0.06 milligram medetomidine hydrochloride (Domitor®) per kilogram bodyweight. The medetomidine was reversed between 40-60 minutes after darting with 0.3 milligram atipamezole (Antisedan®) per kilogram bodyweight.

Each animal was also injected with procaine penicillin and benzathine penicillin (Duplocillin®) at 15,000 IU per kilogram bodyweight to avoid infections from the dart wounds.

Hair of four regions were collected; neck, belly, left thigh and tail. The hair of the aardwolf was clipped with normal scissors as close to the skin as possible. The collected hair samples were stored in plastic vials and labelled with collection date and name of the animal.

Washing

Previous studies with hair samples have shown that sometimes false positive hormone concentrations can be measured due to external contamination of the hair *[Davenport et al, 2006]*. For this reason, hair samples were washed to remove fat and possible external contamination. The samples were washed with a solution of 1:100 warm tap water and Johnson baby shampoo® (see appendix 2). The sample and the solution were shaken for one minute. Nine minutes later the samples were rinsed five times with tap water. The samples were subsequently dried overnight at room temperature.

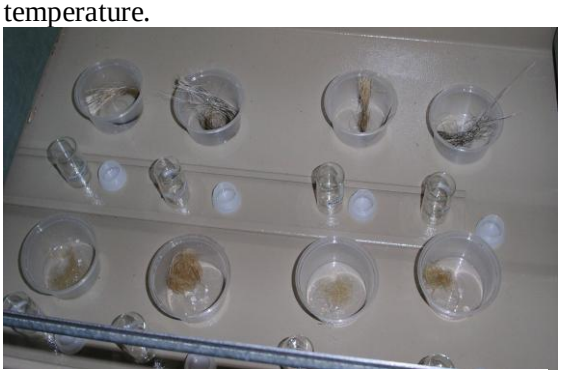

Figure 4. Washed samples drying overnight.

Preparation

In the sterile laboratory the collected hair samples were accurately clipped in lengths of 2cm pieces. The hair were divided in specific regions; beginning-2cm, 2-4cm, 4-6cm, 6-8cm, 8-10 and 10 until the end. Precisely 20mg hair was weighed of each sample and the hair were minced in 3-4mm pieces. The minced samples were placed in a vial. All the time the hair were handled with sterile gloves and instruments.

Extraction

The extraction protocol was used as described by Koren et al, 2002. One millilitre ethanol (80%) was added in the glass vials and the vials are sonicated for 30 minutes. The vials are incubated overnight at 50°C with gentle shaking. The solutions were pipetted off into glass tubes, evaporated and reconstituted with phosphate-buffered saline (PBS; ph=7).

EIA

Methodological similar assays were carried out as described by Ganswindt et al., 2002, 2003. In brief, 50 ml of the obtained hair sample solution was pipetted in duplicate into the microtiterplate wells. Subsequently, 50 ml of biotinylated steroid label and antiserum were added and the plate incubated overnight at 4°C. The plates were washed four times and 150 ml (20 ng) of streptavidin-peroxidase was added to each well. This was followed by an incubation in the dark for 30 min. Afterwards, the plates were washed again before 150 ml peroxidase substrate solution was added. The plates were further incubated for 30–60 min until the reaction was terminated by adding 50 ml of 4N H2SO4. The absorbance was measured at 450 nm.

Expected results

To validate the results of this study, the mean hormone concentrations are compared with the hormone concentrations found in the faecal samples of the same animal in the same period. These samples are collected in a parallel study.

Accorsi et al, 2008 used the same extraction protocol and found a significant positive correlation in domestic dogs and cats between the concentrations of cortisol determined in hair and faeces. In this study the same correlation for levels of different hormones is expected to be measured.

No significant difference is expected to be found between the concentrations found in the hair of the different regions of the aardwolf. The different hair types are expected to have the same growth rate and the same way of nourishing. Therefore it is expected that it is possible to use each type of hair for endocrine measurement in aardwolves.

Different hormone concentrations are expected in different regions of one hair. Due to the continuous growth of mammal hair, the long term fluctuations in haematogenous hormone levels will be reflected in the concentration measured in the different parts of one hair. Further research would be necessary because there are virtually no related comparable researches.

Due to differences in gender metabolism, the measured testosterone levels will be significantly higher in the males than in the females. The opposite can be expected for estrogens levels.

Discussion

The main purpose of this research was to develop a non-invasive technique for measuring hormone concentrations in hair samples. However, in this study samples were collected by sedating the study animals and thus it has not been established yet as a non-invasive way to collect the necessary samples. However, these initial samples were collected under sedation and serve as samples to validate the assay method before it can be implemented as a non-invasive method.

Several wash techniques are described in the literature. Water is expected to be the safest medium, but Eser et al, 1997 writes that water penetrates hair, increasing the risk of extracting steroids from more locations within the interior of the hair shaft. Until now, it is unknown if water extract hormones in hair. In this study different hormone levels are expected in different regions of one hair. However, other studies suggest different outcomes. Cortisol concentrations do not differ between the proximal and distal portions of the hair shaft *[Davenport et al,2006; Yang et al,1998]*. In humans the levels of steroid hormones in hair do not vary significant between different regions of the scalp. *[Wheeler et al,1998]*

As far as hair is concerned, no other references for this species are found in the literature. There is very little known about the endocrinology of this species, therefore the results were compared with the faecal concentrations.

Assuming that it is possible to measure longitudinal hormone profiles within one hair, the long tail and neck hair are the best hair types to use for this purpose is because of the length of these hairs.

Expected conclusion

Hair collection can be expected to be used for non-invasive endocrine measurement in aardwolves. It can be expected that hair reflects a long term profile of androgens, progesterone and glucocorticoids levels. Daily fluctuations in blood levels are expected to have no influences on the hormone

concentrations in the hair and therefore hair probably only reflects longterm profiles. Each type of hair can possibly be used for hormone measurement in aardwolves.

Very little literature is written about hair sampling and hormone measurement. There is also little known about the endocrinology of the aardwolf. Therefore more research should be done before hair sampling is a good useful non-invasive technique for hormone measurement in aardwolves.

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Appendix 1

Table 1. Differences between non-invasive methods, M. Engelkes

	Long term hormone profile	**Impact acute stress respons**	**Measurement daily fluctuations**	**Storage / Transport**	**Collecting for wildlife research**	**Other**
Feacal	Multipele sampling	Insensitive	Yes	Frozen	Useful	
Feathers	Single sample	Insensitive	No	No specific requirements	Useful	Only birds
Hair	Single sample	Insensitive	No	No specific requirements	Useful	
Milk	Multipele sampling	Sensitive	Yes	Frozen	Difficult	Only lactating animals
Nails	Single sample	Insensitive	No	No specific requirements	Useful	
Saliva	Multipele sampling	Sensitive	Yes	Frozen	Difficult	
Sweat	Multipele sampling	Insensitive	Yes	Frozen	Difficult	
Urine	Multipele sampling	Sensitive	Yes	Frozen	Difficult	

Appendix 2

Johnson baby shampoo®
Ingredients:

Aqua, coco-glucoside, sodium, lauroamphoacetate, sodium laureth sulfate, citric acid, polysorbate 20, PEG-80 sorbitan laurate,e, PEG-150 distearate, polyquaternium-10, sodium benzoate, parfum, CL47005, CL 15985