Faecal research as a method to study the physiological health of the African Painted Dog (Lycaon pictus)

Author: Marit de Gelder, MSc student faculty of Veterinary Medicine, Utrecht University, The Netherlands
Supervisor at the faculty of Veterinary Medicine, Utrecht University, The Netherlands: Prof. Dr. Saskia S. Arndt

Table of contents

Abstract

The African painted dog (Lycaon pictus) is an endangered, sub-Saharan living, carnivore species. Very little is known about the physiological health of this species. Therefore, more research is needed. To prevent interfering the pack, a non-invasive method to study the physiological health of African painted dogs is preferred. This study focuses on the conservational advantages of a non-invasive method, faecal research, under field conditions. Questions about stress, reproduction and disease can be answered using faecal research. A detailed overview of parameters that can be isolated from faeces is given. Also, evidence based protocols are included to perform these analysis under field conditions at conservational projects such as Painted Dog Conservation (PDC), Zimbabwe. Since many of these projects rely on private funding, cost-effective strategies are needed. A lot of these analysis, however, still need validation for African painted dogs so further research is necessary. However, by introducing faecal research and monitoring as a standard procedure at PDC, much more information about African painted dogs can be obtained which, in the end, might result in a healthier and growing population of African painted dogs.

Introduction

The African painted dog (Lycaon pictus) is an endangered carnivore species belonging to the family of Canidae (IUCN Red list classification C2a). They live south of the Sahara and there are approximately 6600 adults left in 39 subpopulations (figure 1) (Woodroffe and Sillero-Zubiri 2012). Their pack consists of an unrelated alpha male and alpha female, subdominant non-breeding relatives and the offspring of the dominant pair. However, occasionally subdominants succeed in producing offspring. The sibling females often migrate to form new packs with other males (Girman et al. 1997). The main prey species are Impala, Greater kudu, Thomson's gazelle and Common Wildebeest (Woodroffe and Sillero-Zubiri 2012).

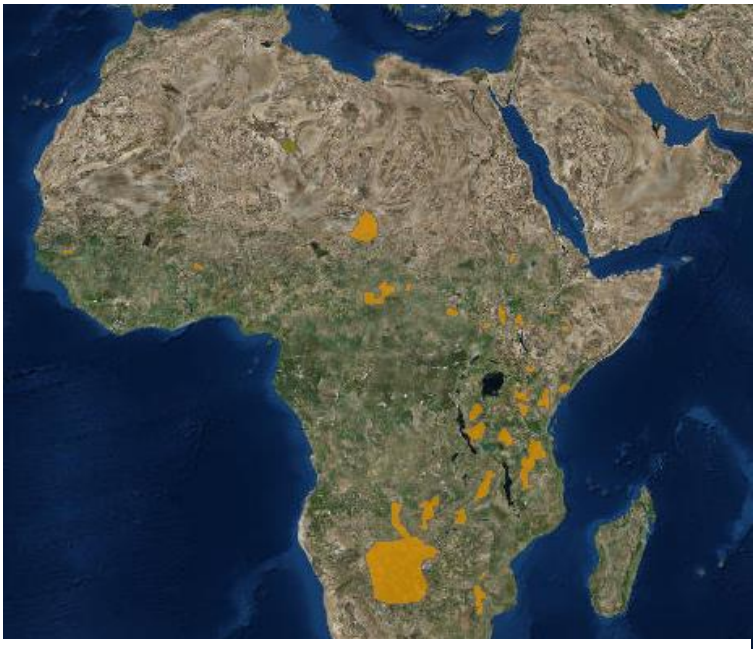

Figure 1: Current range (yellow spots) of African painted dogs in Africa. (Woodroffe and Sillero-Zubiri 2012)

There has been a decline in the number of Painted Dogs in Africa for the last 30 years. This is due to multiple factors. The major causes of the decline are habitat fragmentation, contact with human activity and disease. African painted dogs have home ranges up to 2460 km^2. Road deaths, accidentally getting trapped in snares and persecution by landowners are responsible for 60% of the adult mortality. This is why African painted dogs nowadays persist only in countries with a relatively low human population density. Also disease contributes to the decline of

African painted Dogs (Ginsberg et al. 1997). This shows that action has to be taken to save the African painted dog from extinction.

To achieve this, insight must be gained in the factors that have led to a decline in the number of African painted dogs in certain areas in Africa in the past and the threats for the future need to be determined. There is very little known about the physiological health of wild African painted dogs. Moreover, there is very little supportive data on the influence of health problems and diseases on the decline of this endangered species. This emphasizes the need for a method to study health of African painted dogs across large geographical areas (Van Heerden et al. 1995).

To research the physiological health of a wild and free-ranging animal, the individual should be monitored for a longer period of time. This requires frequently performed physical examinations, blood samples and other diagnostic tools. For this, anaesthesia is required. These frequent anaesthetic procedures are of great disturbance to both the individual and the pack and might lead to a chronic stress response which makes them more vulnerable to disease. To minimize the disturbance to the individual and pack, non-invasive techniques are necessary (Dickens, Delehanty, Michael Romero 2010; Kock et al. 1987). Something that can give a lot of information about the physiological health of a mammal is faecal research. Faecal sample collection is a non-invasive technique and therefore easy to accomplish. It is particularly valuable when researching the long-term physiological processes of an animal such as stress, reproduction and metabolic activity (Behringer and Deschner 2017). Therefore, this study will focus on the conservational advantages of faecal research considering African painted dogs. The focus will lie on the possible faecal study's at Painted Dog Conservation (PDC) in Zimbabwe.

African painted dogs are not just free-ranging, but also kept in zoos or rehabilitation centres. PDC has a rehabilitation facility to house and care for injured or sick African painted dogs. This allows more research possibilities to this endangered species.

The aim of this study is 1) To give a detailed overview of parameters that can be isolated from faeces and benefit to the health, and with that, welfare and conservation of the African painted dog. And 2) to write an evidence based protocol on performing these isolating techniques at Painted Dog Conservation in Zimbabwe. This protocol is based on observations and research in the laboratory on location at PDC.

At PDC, research equipment and financial resources are limited because this organisation relies on private funding. This has been taken into consideration while performing research to laboratory conditions on location that clarified which of these parameters can be studied at PDC in Zimbabwe. With this information, a protocol is written that can be used at PDC and other conservational organisations to extend faecal research to African painted dogs.

Parameters that can be identified in faeces

Glucocorticoids

An adequate stress response is very important for a great variety of things such as welfare, survival, reproduction, immunity and adaptation. Of these, survival and reproduction are of highest importance when it comes to endangered species (Dickens, Delehanty, Michael Romero 2010). An animal's response to a stressor is stimulation of the sympathetic nervous system and the activation of the hypothalamic-pituitary-adrenal (HPA) axis. The result of the HPA axis activation is secretion of glucocorticoid's (GCs). This causes an immediate increase of energy and inhibits physiological processes that are not required for immediate survival such as growth, reproduction and digestion (figure 2). Acute stress has no negative effects on an individual's health and can even have very beneficial effects. Because it is responsible for the fight-flight response and helps the individual to cope with a certain situation of stress it helps the individual survive. However, chronic stress results in chronically elevated levels of GCs which can have some really negative consequences for an animal's health and reproductive state. This can be critical for the survival of a population (Creel 2001; Virgin Jr. and Sapolsky 1997).

Free ranging African painted dogs are exposed to stress caused by social interactions, the threat of predators and humans, and the need of finding food and shelter. With, for instance, increased predator threat, more human interaction and low food availability these factors can lead to chronic stress. Chronic stress can cause elevated levels of GCs for days or weeks. High GC levels act as immunosuppressant's and can therefore have large effects on an individual's health. This can increase the risk of infection and parasitic load in mammals (Busch and Hayward 2009; Sapolsky, Romero, Munck 2000). There is even a negative relationship between high GC levels (indicating chronic stress) and survival (Blas et al. 2007).

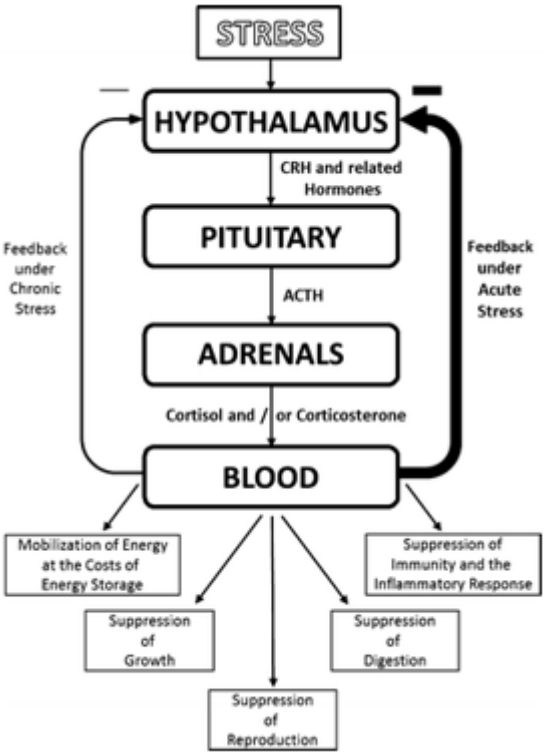

Figure 2: The hypothalamic-pituitary-adrenal (HPA) axis, the effects on homeostasis and negative feedback (Sheriff et al. 2011).

Elevated levels of GCs can also be of great influence on reproduction. As can be seen in figure 2, GCs cause a suppression of reproduction. They inhibit reproductive behaviour and physiology. This will be further discussed in the chapter steroids and reproductive activity (page 6). As mentioned before, the alpha male and female are the only breeding pair of the pack. If one of them is exposed to chronic stress and thus has high plasma levels of GCs, this can have an impact on the continued existence of the pack. For this reason,

it is very important to gain insight into chronic stress of African painted dogs because it can lead to impaired health and reproduction problems .

A small amount of GCs is needed to maintain homeostasis. This is the so-called basal level. GC release differs daily and with life-history cycles. In different mammalian species, a seasonal change in GC release has been found (Busch and Hayward 2009; Landys, Ramenofsky, Wingfield 2006; Michael Romero 2002). The breeding pair of the pack shows higher basal GC levels than the subordinates (Creel et al. 1997). All of these fluctuations should be taken into consideration while studying stress responses in African painted dogs.

Another element that has to be taken into account in the difference between free-ranging and captured individuals. In an enclosed environment it is easier to monitor the fluctuations of GC levels in an individual and draw conclusions, than in free-ranging individuals (Van der Weyde, Martin, Paris 2016). This is due to the fact that in an enclosed environment GC levels can be monitored and measured with a smaller and more standardized interval and therefore help drawing a more reliable conclusion.

GCs are usually measured in blood. However, they can also be identified in faeces as GC metabolites. They reflect the adrenocortical activity over a longer period of time. Therefore, they are more useful to measure chronic stress, caused e.g. by social, biological and/or environmental factors. Therefore, measuring GC concentrations can be of great benefit in ecological and conservational studies (Sheriff et al. 2011). Since the GC levels fluctuate daily, seasonal and per animal, and reproductive status, diet, habituation and gender also influence the GC levels it is important to monitor these levels for a longer period of time to draw conclusions. (Keay et al. 2006). The symptoms of a chronically stressed animal are higher baseline plasma GC levels, acute increases in plasma GC levels following a challenge and an increased amount of time for the plasma GC levels to return to the baseline levels (Dantzer et al. 2014).

GC metabolites are excreted in faeces via the bile ducts. The most common method for analysing these GC metabolite levels is immuno-assay (Keay et al. 2006). The two versions of immuno-assay that are mostly used are Radio Immuno-Assay (RIA) and Enzyme Linked Immuno-Assay (ELISA) (PALME 2005; Sheriff et al. 2011).

Parasitic load

Parasitic infections are a substantial threat to the health and conservation of endangered carnivores. Especially combined with the biggest threats to African painted dogs, habitat fragmentation and contact with human activity, parasitic infections can cause a significant conservational threat (Pedersen et al. 2007). Increased interactions between human populations and African painted dogs due to habitat loss increases the risk of disease transmission between these populations. For example, 21 of the 23 members of an African painted dog pack in Kenya died in 1988 of a rabies infection that was common under domestic dogs in Kenya at that time (Kat et al. 1996). Research on the occurrence of intestinal parasites showed a great prevalence of intestinal parasites in both domestic and stray dogs in Nigeria, South Africa and Zimbabwe (Anene, Nnaji, Chime 1996; Minnaar, Krecek, Fourie 2002). Even more important, there is very little knowledge among pet owners about the zoonosal risks and the spreading to wildlife

of these infections (Pfukenyi 2010). Therefore, research to parasitological infections among African painted dogs might give a decisive answer to the influence of habitat loss and human activity on the physiological health of the African painted dog. This could reveal the possible threat of parasitic load to the conservation of these endangered species.

Another factor that has an influence on parasitic infections is welfare. Captive African painted dogs that experience chronic stress due to management issues for example, will have high basal GC levels. As written before, this leads to an increased risk on parasitic infections. This is caused by the immunosuppressive effects of stress on an individual (Sapolsky et al. 2000, Busch and Hayward 2009). Thus, animals who live in reduced welfare conditions, are at increased risk of parasitic infections. Clinical signs of parasitic infections in both wild and domestic dogs include gastrointestinal disease, decrease of body condition and anaemia (Adolph et al. 2017). Although wild carnivores carry a great diversity of parasite species with them, they often don't show any symptoms in contrast to domestic animals. The reason for this is not completely understood. This might be the result of immunity by the host or they might just show less symptoms then domestic animals do. The host may have established a balance between the parasites and host immunity (Pedersen et al. 2007). This balance however, can be disturbed by other physiological conditions that suppress the immune system such as stress or other diseases. As a result, the parasite load grows and the animal starts showing symptoms of a parasitological infection (Lafferty and Gerber 2002).
This suggests that also in captive African painted dogs parasitological research can have beneficial effects. The most common analysis to examine the presence of helminthic eggs and larvae is faecal examination.

Giardia duodenalis is a common protozoal intestinal infection in mammals and humans worldwide. Giardiasis is associated with severe gastrointestinal disease. Many wild species have been documented as G. duodenalis hosts. Giardia can be transmitted from animal to human by direct contact or indirectly through a contaminated environment. Due to this zoonotic aspect, G. duodenalis is of major public health concern (Thompson 2004). Previous studies showed a prevalence of 26% in wild African painted dog populations (Ash et al. 2010). Due to the increased interaction between humans, domestic dogs and African painted dog populations the risk of disease transmission between these populations is increasing. This has already been seen with rabies and canine distemper infections (Alexander and Appel 1994).

Steroids and reproductive activity

Just like some other carnivores (grey wolves and dwarf mongooses), African painted dogs are cooperative breeders. The alpha male and female almost exclusively produce offspring, while the other members of the pack help rearing the pups (Girman et al. 1997). It is not completely clear what causes the suppression of breeding in the subordinate females. The suppression of breeding behaviour is possibly due to the dominance of the alpha female. Van Heerden et al. found a suppression of ovulation in the subordinate females and suggests that this might be stress-induced. GCs released during stress decrease GnRH release from the hypothalamus and consequently decrease pituitary luteinizing hormone (LH) release. GCs also reduce the responsiveness of the gonads to LH and reduce the number of LH receptors. This leads to a

suppression of reproduction and reproductive behaviour (Sapolsky, Romero, Munck 2000). However, the exact hormonal strategy is not completely understood (Van der Weyde et al. 2015a).

In stable packs, the alpha female gives birth once a year. This suggests that African painted dogs are mono-oestrus, they experience oestrus only once a year. The female cycle of African painted dogs exists, just like domestic dogs, of a pro-oestrus, oestrus, di-oestrus and anoestrus (figure 3). Oestrus occurs between late January and early May (Van Heerden 1985). The gestation period takes 69-73 days (Newell-Fugate, O. Nöthling, J. Bertschinger 2012). During the pro-oestrus, oestrogen concentrations rise and males have increased interest in the female. This is followed by the oestrus. There is a fall in oestrogen concentrations and a rise in LH that triggers ovulation. During this oestrus period, the female allows mount and breeding. Duration of pro-oestrus and oestrus is approximately 14-20 days (Van Heerden 1985). Pro-oestrus and oestrus are followed by di-oestrus. In pregnant females this takes 62-64 days, in non-pregnant females 49-79 days. During this period progesterone rises and falls over this stage. During this period, it is possible to observe pseudo-pregnancy. In this case there is mammary development without pregnancy. The di-oestrus is followed by a period of anoestrus which takes 1-8 months and is considered as a time of reproductive quiescence. During this period follicle stimulating hormone (FSH) is relatively elevated throughout (Root Kustritz 2012).

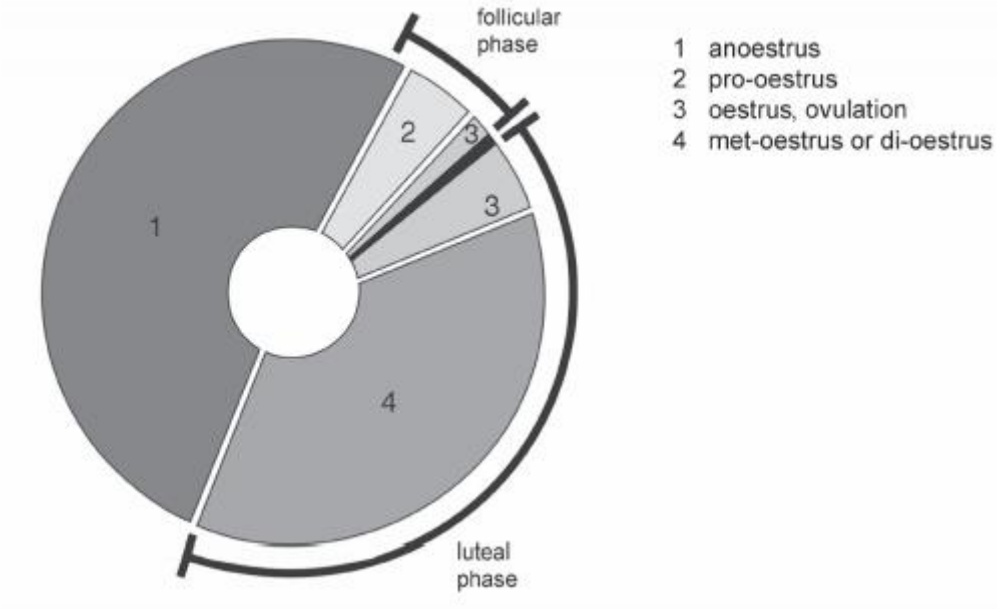

Figure 3: The reproductive cycle in dogs (Rijnberk and Kooistra 2010)**.**

Not everything is clear about the oestrous cycle in African painted dogs, especially in the subordinate females. This is due to limited sample size, studies that exclusively focused on males and captive animals or logistical issues such as sample collection and analysis during field studies (Creel et al. 1997). Therefore, more research to the oestrous cycle and reproductive behaviour of African painted dog females should be done. Gathering reliable information on

reproduction in free-ranging African wild dogs requires a non-invasive method. Measuring faecal steroid metabolites is a great non-invasive method to assess reproductive activity in carnivores. The steroids that can be measured in faeces are oestrogen and progesterone metabolites. Faecal steroid analysis is mainly used to assess female reproduction (Reichert-Stewart et al. 2014). In carnivores, oestrogens are mainly excreted via faeces (58,9%), with a peak extraction 18 h after oestrogen entering the bloodstream. Therefore, faecal oestrogen analysis is a reliable indicator for pregnancy diagnosis and ovulation (Schwarzenberger et al. 1996).

Metabolic activity

Thyroid hormones (T3 and T4) can be used to measure the metabolic activity of an animal. Thyroid hormones are critically involved in the metabolism of animals and can therefore measure the energy balance. In addition, they have influence on body temperature regulation, heart rate and blood pressure. T3 is the more potent thyroid hormone and is being excreted via bile in faeces (Figure 4). The basal metabolic rate (BMR) is a measure for an animal's cellular activity in rest. When nutritionally stressed, vertebrates reduce BMR to save energy. This is supported by a decrease in T3 blood levels. On the other hand, animals increase BMR during low temperatures to generate metabolic heat. This is supported by elevated T3 blood levels. Thyroid hormones appear unaffected by stress (Cristóbal-Azkarate et al. 2016; EALES 1988). This suggests that monitoring of thyroid hormones is a useful method to help determine why and if an animal is in shortage of food. Also, the effects of environmental changes on the energy budget and hunting behaviour of an animal can be studied using this method. Environmental changes and prey availability changes might be due to human interactions. It is essential to understand how animals allocate their energy between competing demands in relation to their environment (Schaebs et al. 2016). Also, the effects of climate change on an individual can be measured. An individual's thermal neutral zone is the zone where no facultative thermogenesis or heat dissipating mechanisms are needed. A decrease in thyroid hormone is seen when temperatures rise above the individual's thermal neutral zone and increase when temperatures fall below this zone (Wasser et al. 2010). Also, an elevation of T3 level prior to the mating season is seen, increasing the intensity of mating activity. Thyroid hormone metabolites can be measured in faeces and therefore provide reliable information about metabolic activity, nutritional status and thermogenesis (Cristóbal-Azkarate et al. 2016; Schaebs et al. 2016; Wasser et al. 2010). In particular information about the nutritional status of an individual can be of great use in conservational studies. (Wasser et al. 2010) showed that

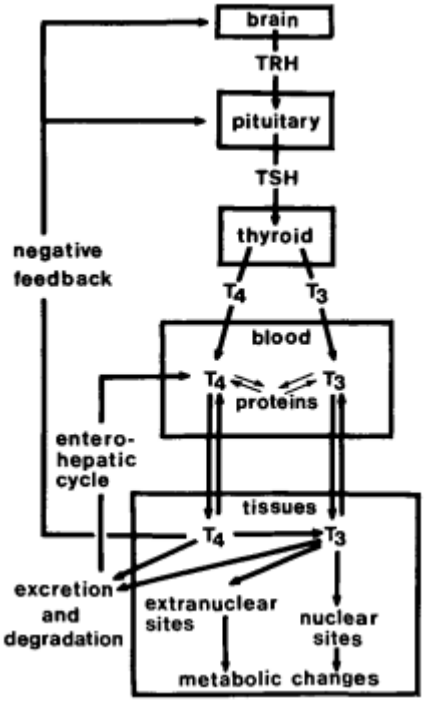

Figure 4: Thyroid pathway and function in mammals (EALES 1988)

thyroid hormone metabolites can be reliably measured in faeces in dogs. This suggests that the same analysis can be applied to African painted dogs.

Viral infections

Human populations are ever expanding. Subsequently, contact between wild and domestic animals increases. Because they have very large home ranges, the African painted dog often leaves the protected reserve area and comes into contact with domestic dogs. The domestic dog is a close relative to the African painted dog, for that reason this contact can cause transmission of pathogens between these species. This makes it more important to monitor domestic species that are in close contact with their endangered relatives. Also, disease surveillance is recommended. Previous studies have already demonstrated the influence of increasing contact between domestic and wild dogs, on disease outbreaks and mortality in African painted dogs (Alexander and Appel 1994; Kat et al. 1996; van de Bildt et al. 2002).

The following viruses are able to infect domestic and African painted dogs and can be detected in faeces. In the past, these viruses have caused large outbreaks and high mortality. Monitoring of the presence of these viruses in African painted dog populations is therefore recommended.

Parvoviridae

Canine parvovirus (CPV2) is a virus belonging to the family Parvoviridae. CPV2 is a non-enveloped DNA virus (Zhou et al. 2017). CPV2 infection is an acute enteric disease in dogs with high morbidity and mortality. Symptoms in puppy's are vomiting, diarrhoea, depression, dehydration and fever. It mostly occurs in young dogs from 6 weeks up to 6 months of age. Infection and transmission is primarily accomplished via the faecal-oral route. Large numbers of virus are shed via faeces and the disease has an incubation period of 4-7 days. Shedding of the virus may continue 3-4 weeks after showing symptoms. Canine parvovirus has a survival rate as low as 9.1% without treatment (Goddard and Leisewitz 2010a). Virus particles can be demonstrated by electron microscopy. EIA can demonstrate viral antigen in faeces and multiplex real time PCR can detect viral DNA in faeces (Quinn, P. J., Markey, B. K., Leonard, F. C.,, FitzPatrick, E. S.,, Fanning, S.,, Hartigan,P.J.,, 2011).

Paramyxoviridae

Canine distemper is an acute and contagious disease in dogs and wild carnivores caused by the canine distemper virus (CDV). CDV is an envelope containing RNA virus belonging to the family Paramyxoviridae. Canine distemper is characterized by high mortality in dogs and African painted dogs and outbreaks of the disease have already been reported in African painted dogs (Alexander and Appel 1994; Goller et al. 2010; van de Bildt et al. 2002). Transmission is accomplished via direct contact or aerosols and domestic dogs are a primary reservoir for the virus. There is a rapid spreading of the disease among young dogs between 3-6 months of age. The incubation period is 1 week and the virus is excreted 60-90 days post-exposure. Acute disease results in either recovery and life-long immunity or the development of neurological signs and eventually death (Alexander and Appel 1994; Quinn, P. J., Markey, B. K., Leonard, F. C.,, FitzPatrick, E. S.,, Fanning, S.,, Hartigan,P.J.,, 2011).

To identify CDV in faeces, a polymerase chain reaction (PCR) can be used. PCR requires specific primers of the DNA that to be amplified and can detect viral pathogens. CDV is a RNA virus and therefore reverse transcription-PCR is used. With this method RNA is first transformed to cDNA using a RNA primer and the enzyme reverse transcriptase. The cDNA is then amplified by PCR with primers complementary to the two ends of the molecule. Then the products are analysed with gel electrophoresis. This can tell if the RNA of interest is present (Russel 2014).

Materials and methods

Overview of faecal parameters that can be measured in faeces and their analyses

Parameter	Analyses
Glucocorticoid levels (page 10)	Enzyme- immunoassay (Goymann et al. 1999) Radio- immunoassay (Young et al. 2004)
Parasitology (page 11)	Centrifuge-sedimentation-flotation method (Markovics and Medinski 1996). McMaster flotation (Taylor, Coop, Wall 2007)
Progesterone metabolites (page 13)	Enzyme-immunoassay (Schwarzenberger et al. 1996) Radio-immunoassay (Schwarzenberger et al. 1996)
Oestrogen (page 13)	Enzyme-immunoassay (Reichert-Stewart et al. 2014) Radio-immunoassay (Van der Weyde et al. 2015b)
T3 and/or T4 (page 14)	Enzyme-immunoassay (Wasser et al. 2010) Radio-immunoassay
Canine Parvovirus (page 17)	Faecal Snap Test (Proksch et al. 2015)
Giardia duodenalis (page 17)	Faecal Snap Test (Rishniw et al. 2010)

Measuring GC levels in faecal samples

Sample collection

Since GC levels fluctuate daily and seasonal it is important to collect the samples at a specific time of day or time of year. It is important to preserve faecal samples as soon as possible after defecating to minimize the chance of microbal degradation. After collection, the samples need to be frozen at -20^{0}C immediately, but if that's not possible for instance in field studies, they can be placed in a container upon wet ice until permanently frozen. The samples should be analysed within a maximum of 120 days (Khan et al. 2002). The most commonly used method for shipping is the use of dry ice (Hunt and Wasser 2003). Also it is recommended to homogenize the sample before extraction (PALME 2005; Van der Weyde et al. 2015b; Van der Weyde, Martin, Paris 2016). .

Extraction

Before analysing the GC metabolites in the sample, the hormones must be extracted from the faeces. For the extraction procedure a method is used previously described by (PALME 2005; Van der Weyde et al. 2015b).

- Put 0,5 g of faecal sample in a plastic tube and homogenize the sample
- Add 5mL of 80% methanol to the sample and shake/vortex
- Centrifuge the tubes for 20 minutes (4^0C, 3000 x g).
- Collect the supernatant in a tube.
- Tubes can be capped and stored under -20^0C until assay

Analysis

For the analysis there are two frequently used options; radio-immunoassay (RIA) and enzyme-immunoassay (EIA). The advantage of EIA is that no radioactive material is needed and it is possible to develop group-specific antibodies to detect GC metabolites in faeces (Sheriff et al. 2011). The corticosterone assay shows a clear response after administration of ACTH, the hormone that stimulates the adrenals to produce GCs, and therefore a common corticosterone EIA kit can be used (Goymann et al. 1999).

RIA however, is more specific. A double-antibody RIA for corticosterone is a common assay to analyse GC metabolites in faeces and a corticosterone RIA kit can be used. However, in field studies, the use of radioactive material might be restricted so therefore EIA is an easier assay to perform (Young et al. 2004). For Painted Dog Conservation it is recommended to use an available EIA in a laboratory nearby since this is more cost-efficient than starting up an own EIA in the lab. Further on, a more detailed overview of both RIA and EIA will be given (see page 15, immunoassays).

Parasitology

Sample collection

Faecal samples must be collected as soon after defecation as possible with a maximum of 48 hours after defecation. Samples must contain as little debris as possible. The samples can be collected and kept in sealed and labelled plastic bags or plastic vials with screwcaps before analysis (Lynsdale et al. 2015). Therefore, it is very useful to keep a 'collection kit' containing gloves and plastic vials in the cars so all useful samples can be collected immediately. Since eggs hatch rapidly, it is preferred to perform the analysis directly after the collection fresh faeces. If that is not possible, the sample can be refrigerated until analysis for the maximum of a week. Another option to preserve faeces is to put 5 g of faeces in 15 ml of 10% formalin, however the refrigerating method is more reliable and therefore preferred (Crawley et al. 2016).

Analysis

Before analysing, the samples should be macroscopically examined on the presence of cestode proglottids or adult helminths. The following methods can be used to detect the presence of eggs or larvae in accordance to (Taylor et al. 2007, Great Britain., Ministry of

Agriculture,Fisheries and Food., 1986). When analysing the slides under the microscope the following features should be taken into consideration: Egg size and shape, shell thickness and structure, presence of embryo's. Information and pictures on egg features can best be used when analysing the samples to determine the eggs found. These references can be found in Taylor et al. 2007, page 262-268.

Centrifuge-Sedimentation-Flotation (CSF) method

This method allows the eggs to separate from the faecal sample and float upon the surface. It combines both sedimentation and flotation and has therefore more reliable results than a single sedimentation or flotation test. Also, it makes the use of both sedimentation and flotation methods separately unnecessary. Separating the eggs occurs by suspending the sample in a liquid with a specific gravity higher than that of the eggs. A different specific gravity is needed for different eggs.
Nematode and cestode eggs float in a liquid with a specific gravity 1.10-1.20. Liquids based on sodium chloride or magnesium sulphate can be used.
Trematode eggs float in a liquid with a specific gravity 1.30 – 1.35. Liquids based on Zinc Chloride, zinc sulphate or a sugar solution can be used.

Centrifuge-Sedimentation-Flotation method (Markovics and Medinski 1996)

1. Add 2-3 g of faecal sample to a cup.
2. Add approximately 25 ml of water and make a suspension.
3. Pass the suspension through a sieve and check sieve for possible proglottides.
4. While mixing, pour the suspension in a test tube.
5. Centrifuge for 2 minutes at 3000 rpm.
6. Pour of the supernatant slowly.
7. Fill 2/3 of the test tube with the flotation solution and make a suspension.
8. Centrifuge suspension at 3000 rpm for 2 minutes.
9. Add more flotation solution until a meniscus is formed.
10. Place a cover slip on top of the meniscus.
11. Leave standing for 15 minutes.
12. Remove cover slip from the test tube by lifting straight upward and place on a glass slide.
13. Examine under microscope at low power.

This CSF method is adapted to the laboratory at Painted Dog Conservation. This lab did not contain a swing-centrifuge, but a fixed-angle centrifuge. This makes it impossible to centrifuge the test tube with a cover slip on top of the meniscus. Leaving it standing for 15 minutes allows the eggs to float to the surface and attach to the cover slip.

The CSF method is a qualitative method to examine faeces on parasites. However, in some cases it is useful to perform quantitative techniques where it is desirable to count the number of eggs or larvae present in a gram of faeces. This technique consists of a counting chamber filled

with faecal sample and a salt solution. The eggs will start floating like during the floatation method.

McMaster flotation

- Suspend 3 g faeces in 42 ml saturated salt solution (specific gravity 1.20)
- Pour suspension through a fine mesh sieve (205µm)
- Collect the filtrate and fill a 15 ml test tube
- Centrifuge for 2 minutes at 2000 rpm
- Pour of supernatant and fill tube to previous level with flotation solution
- Mix contents of tube by inverting it six times
- Remove fluid with pipette and fill both chambers of the McMaster slide
- Count all the eggs in both chambers
- The number of eggs per g faeces is the total number of eggs in both chambers multiplied by 50.

At Painted Dog Conservation the flotation method was used to identify parasite eggs in faeces. This method however, did not give a clear view, but a rather cloudy view because there was a lot of faecal debris that came along with the eggs. This made it very hard to properly identify the eggs in the sample. To get more reliable results and to get rid of all the faecal debris on the slide, the CSF method was introduced as written above.

Instead of the formerly used Zinc-sulphate, a sugar solution was introduced because of the following reasons. First, Painted Dog Conservation is a non-governmental organisation relying on donations. A sugar solution is easy to make and the costs are relatively low in contrast with Zinc-sulphate. Second, regarding the fact that all waste is being dumped into the environment, a sugar solution has less impact on the environment then Zinc-sulphate. Third, just like Zinc-sulphate, a sugar solution can separate all eggs from debris.

To make the sugar solution, 454 g of sugar was dissolved in 355 mL of hot tap water. Then the weight of the solution was measured to verify if the specific gravity was 1.27g/cm3.

Steroids and reproduction

Sample collection

Progesterone and oestrogen are just like corticosterone steroid hormones. Therefore, the same techniques used for the analysis of GCs in faeces can be applied to the analysis of progesterone and/or oestrogen. Since the hormones progesterone and oestrogen fluctuate seasonally it is important to collect samples at approximately the same time of day or time of year. During sample collection it is important to keep track of the clinical signs of oestrus, such as female receptivity and mating behaviour, to compare those with the findings of the hormone analysis. Freezing at -20 degrees Celsius is the best method to store the samples until further assay (PALME 2005; Van Heerden 1985).

Extraction

For the extraction of progesterone and/or oestrogen metabolites the same method as for extracting GC metabolites can be used (PALME 2005; Van der Weyde et al. 2015b).

Analysis

Unconjugated oestrone and oestardiol-17α or -17β can be measured using RIA or EIA (Reichert-Stewart et al. 2014; Van der Weyde et al. 2015b).

Progesterone is not present in faecal samples, therefore progesterone metabolites are measured. With this analysis pregnancy, abortion, corpus luteum function and seasonality can be monitored (Schwarzenberger et al. 1996). Progesterone metabolites can be assayed with a commercial progesterone RIA or EIA kit. Progesterone is metabolised in 5α- and 5β-pregnanediones which are present in faeces. Therefore, antibody's should be raised against these two metabolites instead of progesterone.

Metabolic activity

Sample collection and storage

Samples need to be collected as soon after defecation as possible. Samples must be collected in plastic tubes, homogenized and stored at -20 ^{0}C prior to analysis (Wasser et al. 2010).

Extraction

For extracting and analysing the faecal samples, a method used by (Wasser et al. 2010) can be followed.

1. Homogenize samples and freeze dry prior to extraction.
2. Add 15 mL of 70% ethanol to 0.1 g faecal powder.
3. Vortex for 30 minutes at 1 pulse/s.
4. Centrifuge for 20 minutes at 2200 rpm.
5. Poor of supernatant and store in airtight tube.
6. Repeat procedure with the left over faecal pellet.
7. Combine the resulting supernatant with the first.
8. Store ate -20 C until assay .

Analysis

To analyse faeces on T3 and T4 both EIA and RIA can be used. Total T3 and T4 commercial EIA and RIA kits are available (Wasser et al. 2010).

Immunoassays

There are two forms of immunoassay: enzyme immunoassay (EIA) and radioimmunoassay (RIA). Both work on the same principle, they are competitive binding assays that bind an antibody against a certain part of the molecule of interest (antigen). In RIA an antibody against

the antigen is labelled with a radioactive isotope (figure 5). This generates a radioactive signal to measure antibody binding. In EIA an enzyme is linked to the antibody (figure 6). Binding of the antibody to the antigen can be detected by a change in colour of the substrate (Murphy 2012).

There are two variants of these immunoassays. Non-commercial assays can be developed in laboratories. The specific antibody must be made or purchased. This is especially useful when large numbers of samples are being processed. The other variant is a commercial kit for either RIA or EIA. They have been adapted for wildlife studies and usually contain all the reagents necessary. However, they have not been validated specifically for African painted dogs and therefore will need further validation (Murphy 2012; Sheriff et al. 2011).

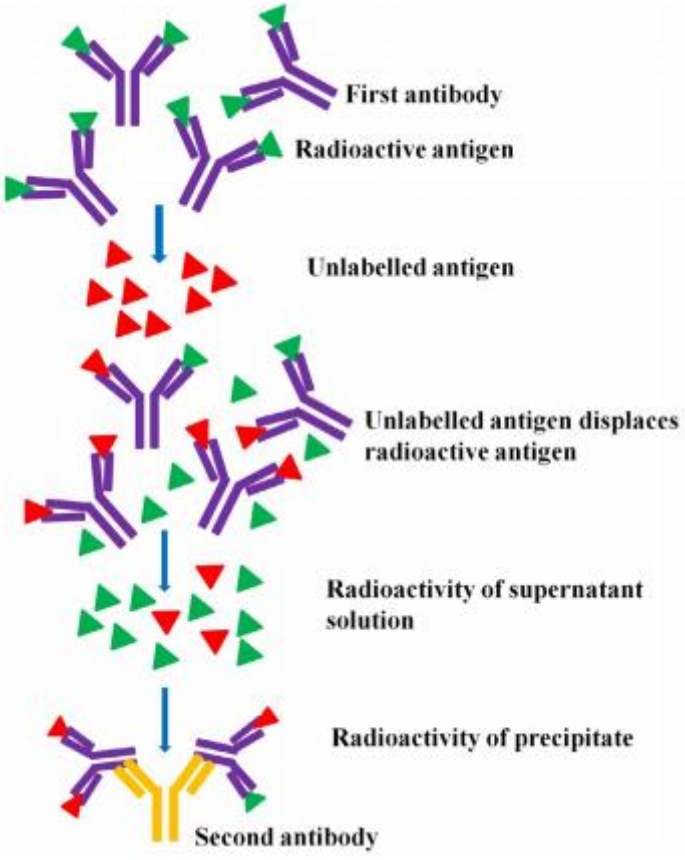

Figure 5: Radio Immunoassay (RIA); antibodies are labelled with radioactive antigen.

(Ranjan, Minakshi, Prasad 2015)

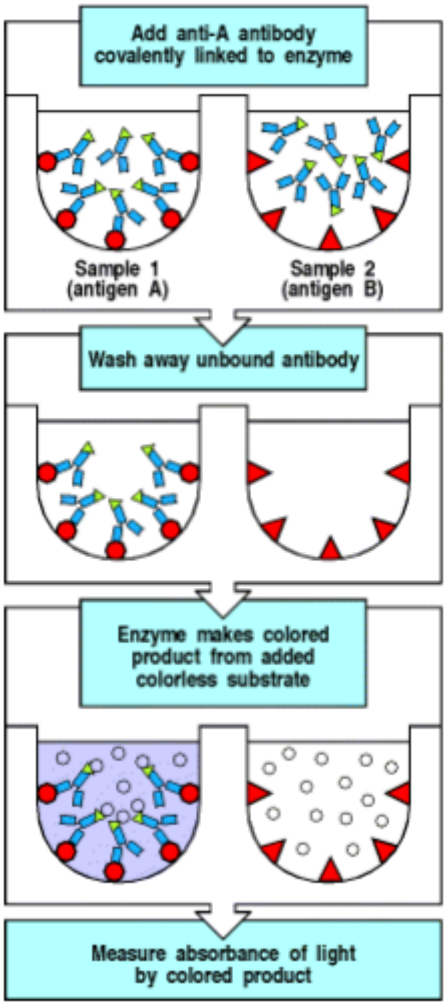

Figure 6: Enzyme Immunoassay (EIA); antibodies are linked to enzyme. Substrate is added to colour the product. (Murphy 2012)

The relatively small amount of samples that need to be analysed at Painted Dog Conservation, do not justify the costs of the development of an extensive laboratory that can handle radioactivity at PDC. Therefore it is recommended to use a commercial RIA or EIA kit or to transport the samples to a more extensive and experienced laboratory.

GC identification

If a RIA is used to identify GC metabolites in African painted dog faeces a corticosterone RIA is most sensitive (Creel et al. 1997; Monfort et al. 1998; Young et al. 2004). If an EIA is used to identify GC metabolites in African painted dog faeces corticosterone EIAs show a clear elevation after an ACTH challenge test (Monfort et al. 1998). Therefore a corticosterone EIA is suitable for identifying GC metabolites (Bashaw et al. 2016; Wheeler et al. 2013)

Progesterone identification

EIA: Progesterone is not present in faecal samples. Therefore antibodies against progesterone metabolites should be raised. These antibodies should identify C20 positions (20-oxo, 20α-OH, 20β-OH) of the pregnane molecule. These 20-oxo-preganes cross-react with progesterone antibodies (Schwarzenberger et al. 1996). Antibodies can be raised against 20-oxo-group containing 5α- and 5β-pregnane metabolites. The EIA is named group-specific for this reason.

Oestrogen identification

In the domestic dog, the ovulation is characterised by a pre-ovulatory increase of faecal androgens. Oestrogen in carnivores is mainly secreted in faeces and therefore measuring the pre-ovulatory increase in oestrogen is a reliable way to indicate ovulation. If an EIA is used, unconjugated oestrone or oestradiol-17α or -17β can be identified in faeces. Specific oestrogen antibodies or unconjugated oestrogen antibodies can be raised for this assay (Schwarzenberger et al. 1996).

Thyroid hormone identification

T3 is the bioactive hormone and is more potent than T4. This makes T3 the more informative hormone. T3 is excreted in faeces in proportion to its use. Therefore total T3 RIA's (Wasser et al. 2010) and competitive total T3 EIA's (Schaebs et al. 2016) are used. However, since faecal thyroid hormone has never been analysed in African painted dogs, it is important to validate the assay before analysis.

DNA Analysis

A lot of information can be obtained from genetic analysis of faecal samples. DNA material can be extracted from faeces and analysed using polymerase chain reaction (PCR). Not just information about the individual itself, but also information about gut passengers such as pathogens and prey can be obtained using specific PCR primers. The following markers can be found in faeces:

- Mitochondrial DNA (mtDNA): The mtDNA cytochrome b gene has little intraspecific variation and is therefore used in species identification. Also mtDNA of the prey can be present in faeces and can be used to obtain information about diet. Also pathogen

sequences can be amplified to identify the presence of pathogens (Kohn and Wayne 1997).

- Microsatellites (simple sequence repeats (SSRs)): Microsatellites are tandem repeats of short sequence motifs. They can be used to obtain a multilocus genotype which is unique to individuals. Therefore, they are used to study kinship and paternity. This can give more information on social behaviour and mating strategies. In addition, identification of an individual is possible with this method (Barrientos et al. 2016; Webster and Reichart 2005).
- SRY gene: Sex determination can be accomplished using PCR primers for the sex determining region Y (SRY) gene on Y-chromosomes (Kohn and Wayne 1997).

Sample collection for DNA analysis (Constable et al. 2001; Wasser et al. 1997)

- Collect 2-10 g of faecal sample in vial
- Completely cover the sample in 100% ethanol
- Store at 4 ^{0}C until further processing

Sample collection for virus identification (Kaur et al. 2016)

- Collect 2-10 g of faecal sample in vial
- Cover sample in phosphate buffer saline
- Store at 4 ^{0}C until further processing

Extraction and PCR analysis

Since the total costs of building a PCR lab are very high, it is more efficient to collect the samples and analyse them in an existing lab if possible.

Faecal snap tests

Canine Parvo virus

The SNAP Parvo test from Idexx Laboratories is a faecal EIA on canine parvovirus commonly used worldwide. It is a low cost in-house method that is easy perform. Therefore it can be of great use at field studies such as performed at PDC. Although the sensitivity and specificity of PCR assays is higher, it is a very expensive and labour-intensive technique and for this reason less suitable for field studies (Proksch et al. 2015).

Giardia duodenalis

G. duodenalis cysts are excreted intermittently. This means that a negative sample does not prove the absence of G. duodenalis in that individual. African wild dogs can be asymptomatic carriers. However, young, injured or stressed animals can start showing symptoms due to immunosuppression. Symptoms that occur during a symptomatic infection are severe loose or watery diarrhoea and in some cases vomiting (Olson, Leonard, Strout 2010). If a major part of the pack starts showing symptoms, the pack is unable to successfully hunt and is in serious danger of extinction.

Recognizing cysts in faecal samples using CSF methods under the microscope is hard due to the presence of debris in faecal samples and the small size of the cysts. For these reasons, G. duodenalis is hard to diagnose by microscopic detection. This requires a high level of expertise and is usually limited to experienced laboratories. This makes detecting G. duodenalis in African painted dogs faecal samples at Painted dog conservation a challenge (Uehlinger et al. 2017).

A very simple commercial G. duodenalis test available is the IDEXX SNAP® Giardia test. This is a quick faecal test based on the EIA principle (see page 15, 'immunoassays'). A positive result indicates that the animal has cysts or giardia trophozoites in the intestine and may be shedding cysts in faeces. All the materials needed are applied by the kit including users instructions and information on how to interpret the results.

Discussion

Most research on the African painted dog focuses on behavioural or ecological aspects. This, however, usually fails to tells us anything about the physiological health of an individual or pack. The aim of this study was to give an overview of parameters that can be isolated from faeces that provide information about the physiological health of the African painted dog. The second aim was to write evidence based protocols to perform these isolating techniques on location at PDC in Zimbabwe. Combining data from behavioural research with information about the physiological health of an individual or pack is much more relevant in a conservational context than behavioural research on its own. With this combination of research, the influence of behavioural and ecological changes on the health and reproduction of an individual or pack can be monitored. And if necessary, an intervention can take place. For instance, the influence of habitat fragmentation on stress levels and with that increased risk of disease and death can be researched. There is a great need for research methods that provide more information about the health and other physiological processes of African painted dogs. Analysis of faecal samples is a non-invasive method and therefore less stress- and harmful for the individual. The protocols written above give an indication of the possibilities on how to monitor health by faecal research. However, most of these analyses are validated for other species, suggesting that it is a possibility, but to validate the analysis for African painted dogs further research should be done.

Research in captive African painted dogs gives more possibilities. It is easier to determine which faeces sample is produced by which individual. In that way conclusions can be drawn on which animal carries certain parasites or viral infections and might form a risk for the other individuals in the enclosure. Also, an individual's behaviour, food intake, and environmental conditions can be monitored more carefully. This suggest that research in captivity might be the first step in establishing baseline hormonal levels for African painted dogs in general. This could be of great benefit to the African painted dogs in the wild.

Another thing that has to be taken into consideration is the monitoring and/or vaccination of domestic dogs. Diseases such as parvo, canine distemper and many parasitic infections can spread from domestic dogs to African painted dogs and vice versa (Alexander and Appel 1994; Goddard and Leisewitz 2010b; Goller et al. 2010). Due to their increasing interaction with

human activity, this forms a potential risk for African painted dogs. For this reason, local domestic dogs should be monitored and/or vaccinated to prevent these diseases from spreading to African painted dogs.

Glucocorticoids

Without the influence of GCs that are released during a stressful event such as darting for taking blood samples, the results of the GC analysis are much more reliable (Behringer and Deschner 2017). GC levels in blood fluctuate during the day due to minor stress events. The time from the stress event to the release of GC metabolites in faeces might compensate for these blood fluctuating GC levels. Faecal GC metabolites give an average of GCs secreted over a specific amount of time. Therefore it might give more information about chronic stress than blood GC levels. Sheriff et al., 2011 suggests that faecal GCs might demonstrate the baseline GC levels in blood. This baseline is extremely important to decide whether an animal is chronically stressed or not. As long as this baseline is not known results from faecal GC analysis should be interpreted with care and environmental and behavioural information should be taken into consideration as well (Sheriff et al. 2011; Van der Weyde, Martin, Paris 2016). Measuring and monitoring faecal parameters can contribute to the conservation and health of the African painted dog. High GC levels indicate (chronic) stress and this might suggest decreased welfare. Therefore, in enclosed environments such as rehabilitation centres or zoos, faecal research on GC levels can provide information about the welfare of African painted dogs.

High GC levels are a physiological response to stress and are important for coping with certain events. This, however, does not mean that the animal is immediately at risk of infection or in a state of poor welfare. To determine if an individual or pack is under the influence of chronic stress, it is important to routinely collect and monitor the GC levels. GC levels can be influenced by seasonal variation, diet, reproductive status, habituation and gender (Keay et al. 2006). For this reason, the results of faecal GC level analysis should always be interpreted in combination with behavioural or seasonal observations.
If a pack shows sudden high GC levels after migrating to a new area with a lot of human interaction or after a traumatic event like pack fragmentation, action can be taken by migrating them to a new area for instance.

Another reason why monitoring GC levels is important is the rehabilitation centre at PDC. The goal of this centre is to take in wounded African painted dogs, help them recover and finally release them in close proximity to their pack. However, does the amount of stress and with that the immunosuppressive effects of GCs help the individual to recover or does it cause the recovery more harm? To answer this question further research should be done. Monitoring GC levels by faecal research could be a useful start. This could be done by comparing the recovery of wounded individuals in a rehabilitation centre and the recovery of individuals with a similar condition in the field with their pack. This is not just a question for PDC but also for other conservational centres.

Parasite load

Wild mammals can carry great loads of parasites without showing clinical symptoms of disease. Therefore, during parasitological research it is important to correspond the results to the individuals' clinical signs. Great parasite loads do not inevitably mean disease and vice versa. Treating an individual without symptoms of a parasitic infection with an anti-parasitic can even result in disease. This is due to the great amount of dead larvae that are migrating from the intestinal wall to the intestine (Pedersen et al. 2007).

Due to habitat fragmentation and expanding human populations, African painted dogs are in closer contact with humans and their domestic animals. This results in a higher risk of parasite transmission between humans, domestic animals and African painted dogs. This is not just a risk for the African painted dog, but also for human populations. African painted dogs can carry large numbers of parasites without showing any clinical symptoms. These parasites are shed in the environment via faeces close to human activity. Some of these parasites, such as Taenia sp. And G. duodenalis, are of great zoonotic risk. For this reason, monitoring parasite loads in African painted dogs is also of benefit to human health (Cleaveland, Laurenson, Taylor 2001). Parasitology is an easy and low-cost method and can therefore be performed on a regular basis. This benefits both the African painted dogs and human health.

Thyroid hormone

Monitoring thyroid hormone can reveal metabolic strategies of African painted dogs. High BMR has been associated in a positive way with survival, growth and reproduction. However in a situation of food shortage, a high BMR can have a negative effect on growth and reproduction because the individual needs more food than an individual with a low BMR. Measuring thyroid hormone and with that BMR might reveal the metabolic strategy of African painted dogs when nutritionally stressed (Burton et al. 2011). Lowered T4 levels in combination with high GC levels are an indication for nutritional stress. Measuring both these parameters provides information about the availability of prey, the efficiency of hunting techniques and the general nutritional status of an individual or pack. Thyroid hormone has been successfully analysed in dog and wolf faeces. This indicates that the same analysis could be used for analysing thyroid hormone in African painted dog faeces. However, since this method is not validated for African painted dogs yet, result should be interpreted with care and further research should be done to validate this analysis for African painted dogs (Wasser et al. 2010).

Reproduction

Previous research has provided a valid method to study reproduction in African painted dogs. With these methods oestrogen and progesterone levels can be measured. This provides information about the reproductive cycle of both the alpha female and the subordinate females. The results should always be aligned with behavioural observations because pseudo pregnant and acyclic dogs have been observed in the past (Van der Weyde et al. 2015b). Information about reproduction of African painted dogs can help captive breeding programmes or might reveal the reason for low birth rates in packs. Also faecal oestrogen and progestogen levels can

identify acyclic and pseudopregnant African painted dogs. (Newell-Fugate, O. Nöthling, J. Bertschinger 2012).
It is also possible to measure testosterone metabolites in faeces. However, it has not been proved that this could be a reliable way to monitor seasonality in African painted dogs (Newell-Fugate, O. Nöthling, J. Bertschinger 2012).

Viral infections

Although it is still unclear what the exact level of threat is, viruses such as CDV and parvovirus have shown to cause disease related mortality in African painted dogs in the past. Concurrent with a canine distemper outbreak among domestic dogs near Masai Mara National reserve, Kenya in 1992, the African wild dogs in that region disappeared. Therefore it is important to identify virus outbreaks as soon as possible so action can be taken (Alexander and Appel 1994; Goller et al. 2010). Individual African painted dogs or packs that show symptoms of parvovirus (bloody diarrhoea and weakness) or canine distemper virus (bloody diarrhoea, neurological signs, coughing) should be examined rapidly. Moreover, if an outbreak of parvovirus or canine distemper virus among domestic dogs in an area close to African painted dogs is identified, further spreading of the virus to wild mammals can be prevented. The faecal SNAP test is a very quick and reliable method to identify Parvovirus in both domestic and African painted dogs. However, identifying Canine distemper virus in faeces using EIA or RIA is a much more difficult, expensive and time-taking process. On the other hand, the high mortality rates and quick spreading of the disease show that it is important to diagnose the individuals when showing symptoms anyway. When a certain pack is diagnosed with the disease, spreading to domestic or other African painted dogs can be prevented. This emphasizes the importance of close contact to a laboratory that performs the analysis and strict protocols on storing and shipping of the faecal samples of individuals suspected of infection. Unfortunately a safe vaccine against Canine distemper virus for African painted dogs does not exist yet, but vaccinating domestic dogs that live in area's close to African painted dogs can prevent spreading of the disease to their wild neighbours.

Snap test

Faecal snap tests on Giardia are inexpensive and easy to use under field conditions. However, previous research showed that this test is able to rule out the diagnosis Giardia but on the other hand, not able to diagnose an individual with Giardia. Since the main symptom of both Giardia and Parvovirus is diarrhoea in young dogs, the SNAP test can be used to distinguish these two diseases. Therefore this could be especially of use when a pack is showing symptoms of disease (Rishniw et al. 2010; Uehlinger et al. 2017).

DNA analysis

DNA analysis can be important for artificial pack formation and for the identification of individual dogs, being released after rehabilitation. It can give information about diet and paternity. however, PCR analysis is an expensive and time taking process. A researcher

experienced in these techniques is needed to lead or perform this kind of research. Therefore, it should be carefully considered if this is a useful analysis to perform at PDC at this moment.

Conclusion

To prevent the African painted dog from going extinct it is essential to do conservational research. Since most research with African painted dogs is done by behavioural studies, little is known about their physiological responses to, for instance, environmental changes and health. This emphasizes the importance of studying physiological parameters of African painted dogs in the field. Combining behavioural research with physiological research makes it possible to study the long-term health of an individual or pack by measuring parameters such as stress, reproduction and metabolic activity. Faecal research is a non-invasive method to study a great variety of these physiological parameters. Most conservational research is done in the field and this requires easy and inexpensive methods to perform these analyses. The protocols described in this paper make a start to this kind of physiological conservational research. The protocols written above give an indication of the possibilities on how to monitor health by faecal research. However, most of these analyses are validated for other species, suggesting that it is a possibility, but to validate the analysis for African painted dogs further research should be done. Due to costs and lack of experience at PDC it is advised to collaborate with an existing laboratory close to PDC to perform more difficult analysis such as EIA, RIA and PCR. However, by introducing faecal research and monitoring as a standard procedure at PDC, much more information about African painted dogs can be obtained which, in the end, might result in a healthier and growing population of African painted dogs.

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