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Faculty of Tropical AgriSciences

Variation of faecal hormones during antler cycle of Père David's deer (*Elaphurus davidianus*)

Master thesis

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Supervisor:
Ing. Radim Kotrba, Ph.D.

Author:
Bc. Zuzana Klapalová

Declaration

Hereby I confirm that I wrote diploma thesis entitled: **"Variation of faecal hormones during antler cycle of Père David's deer (*Elaphurus davidianus*)"** by myself and used only references cited in the text and reported in bibliography.

In Dobříkov on 22nd April 2016

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Zuzana Klapalová

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Abstract

Père David's deer (*Elaphurus davidianus*) further referred as PDD became extinct in the wild and recently reintroduced in China. Therefore, animals are found widely in zoos, deer parks and farms. Male PDD are described to grow is rare cases two sets of antlers in one year. The objectives of this study were 1) to review the methods for determination of hormones from faecal samples and used it for PDD, 2) to evaluate seasonal endocrinology dynamics of antler cycle in PDD through concentrations of faecal metabolites of androgens and 3) to compare the effects of seasonal and antler cycle to hormonal changes in males of PDD. It was hypothesized that concentrations of faecal metabolites of testosterone and epiandrosterone will differ during stages of antler or reproductive cycle, with the highest levels before cleaning of velvet and casting periods as found in other deer species. Faecal samples right after defecation were collected from 10 adult males of PDD from the Institute of Animal Science (IAS) in Prague-Uhříněves and five zoological gardens (Ostrava, Brno, Bratislava, Prague, Chomutov) from 2011 to 2012 in approximately 7-9 days intervals. For processing of the samples, which was realized in the laboratory of the Faculty of Chemistry at the University of Veterinary Medicine, Vienna, species specific method procedure was used. In total, 600 faecal samples were processed. High seasonal dynamics in levels of testosterone and epiandrosterone metabolites was found in PDD throughout the year and the levels of hormone metabolites differed among different stages of antler cycle or reproductive period. The highest levels were found during cleaning of velvet and casting periods. Higher concentrations of hormone metabolites were found in males from zoos. This could be caused by the presence of females and also by the existence of just one adult dominant male in the herd in zoos. In case of only male group, position in hierarchy influenced also levels of levels of testosterone and epiandrosterone faecal metabolites being higher in higher ranking animals. Further research covering also males with double antler cycle would be essential to understand antler cycle hormonal regulation.

Key words: antler cycle, *Elaphurus davidianus*, Enzyme Immunoassay, metabolites of hormones

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Graph 4: Log10 transformed epiandrosterone metabolite levels of all individuals together in different phases of antler cycle or reproductive period. Asterisk marks significant differences (results)

List of the abbreviations used in the thesis

EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
IAS	Institute of Animal Science
PDD	Père David’s deer
RIA	radioimmunoassay

1. Introduction

Père David's deer became extinct in the wild about 100 years ago (Naish, 2011). Today, the deer is found widely in zoos, deer parks, and farms. There is recent reintroduction to small reserves in China. But it does not roam freely in the wild as historically (Miller, 2013). For Pere David's deer it is typical, that males grow their antlers over the winter and cleaning velvet in December or January. They are also known to grow two sets of antlers in one year, but only when intensive feeding is provided (Naish, 2011). This species has antlers with two main beam branched anterior segment, with the points extending backwards (Macdonald, 2001).

Seasonal changes in the levels of sex hormones were already described by several studies in other deer species. Deer in temperate climate are seasonally polyoestrous animals which mean that the sexual cycle is influenced by photoperiod (Goss, 1977). The hormones and neural system are key elements in physiological processes and synchronize the internal functions with the external environment. Hormones are released into the blood by endocrine glands and they are transported into susceptible cells, tissues or organs (Janský and Novotný, 1981). In the final phase they are detected as metabolites, which vary considerably. These metabolites and their quantities are species specific (Palme et al., 2005). To determine hormone in a liquid, specific enzyme immunoassay (EIA) can be used (Muir et al., 2001, Palm, 2005). The advantages of these methods are ease of procedure, low cost and reliability. The main principle of this immunoassay is measuring known hormone which is binding to polyclonal antibody. Starting medium for analysis can be obtained by invasive or non-invasive way and blood, saliva, urine or faecal samples can be used. Recently, non-invasive methods are frequently used, because they cause no physical interference to animal bodies. The range of application of these non-invasive methods is very wide for studying animals in the wild (Strier et al., 1999).

Each EIA technique can be used for a qualitative and quantitative purpose. EIA is used as diagnostic tool in medicine and as quality control measure in various industries. It is also used as analytical tool in biomedical research for the detection and

quantification of specific antigens or antibodies in a given sample (Stephanie et al., 2013).

2. Literature Review

2.1 Père David's deer (*Elaphurus davidianus*)

2.1.1 Origin of Père David's deer

The species of Père David's deer (*Elaphurus davidianus*) evolved in the Pliocene period of the Tertiary, according to fossils excavated in southern Japan (Hofmann, 2007). The Père David's deer were probably found in the lowlands of China, swampy areas and reed-covered marshlands (Nowak, 1999). Population declined due to hunting and land reclamation in the swamp areas as human population expanded (Jiang and Li, 1999) and the last wild animal was shot near the Yellow Sea in 1939 (Bedford, 1951). During the first decade of the 20th century, the 11th Duke of Bedford in the United Kingdom gathered the last 18 individuals of Père David's deer, but only 11 of these deer were capable of reproduction (Bedford, 1951).

Hoffman (2007) informed that the first reintroduction back to China went ahead in the year 1985, when the deer were launched into park Beijing Milu. In 1998 the first group of deer was released from the paddocks into the wider reserve (Hu and Jiang, 2002).

Low number of individuals affect genetic and population strangulation is the reason why species loose big part of their genetic variability. It is possible that the first generation, born in Woburn, descend from only one male which resulted in close relationship to present Père David's deer (Jones et al., 1983).

2.1.2 Description of Père David's deer

The Père David's deer has reddish to deep reddish brown summer pelage with a medial black stripe down the shoulders. Winter pelage is greyish brown with darker areas on flanks and throat. The skin between the hooves is lost (Nowak, 1999). The

unusually long and slender head has large expressive eyes and small pointed ears. The skin around the eyes and the lips is light grey and the neck has a throat mane in males. The legs are long and the hooves are relatively long and slender as an adaptation to walking on soft marshy ground. The tail is ended by a black tuft similar a donkey (Butzler, 1990).

2.1.3 Antlers of Père David's deer

The antlers serve as models for basic research in biology of bones and animal science. It is because they are the only animal bones that are accessible without the intrusiveness of surgical procedures and the potential adverse effect of such procedures, because antlers grow and are casted every year. The antlers are costly sexual secondary characters (Harvey and Bradbury, 1991). Their growth requires a partial demineralisation of the skeleton. This is because the diet cannot supply the enormous amount of minerals required for their rapid growth (Muir et al., 1987). This growth can reach 2-4 cm per day (Goss, 1983). Antler size might not only be an index of male quality, but also of habitat or diet quality. It is inversely related to conditions that exert stress on the physiology of the animal (Ullrey, 1983).

As in majority of other cervid species, only male individuals have antlers (Nowak, 1999). Unique among deer, the antlers of Père David's deer have two main beams branched anterior segment with the tines extending backwards. Another strange feature of the antlers of this species is that there may be two sets per year (double antler cycle), because exceptional growth of antlers in the winter can take place in them (Geist, 1998). The summer antlers are the larger set and are dropped in September after the June-August rut. The second set, if they appear, they are fully grown before end of December and are dropped a few weeks later (Butzler, 1990). New antlers (second set) begins to growth immediately after casting of first pair and their development is finished in May (Huffman, 2001). By Nowak (1999), single pair of antlers is grown and the velvet is cleaned later after January. By Harper (1945), span longitude stems was described to be from 55 to 80 cm. Main beam of antler faces upright and two to three long tines go out of it and they are directed backwards. Lower tine antler is the longest at the end furcate.

It rises impression, as though antlers were pitched conversely, tail forward (Harper, 1945).

As Père David's deer, also the European roe deer (*Capreolus capreolus*) have exceptional growth of antlers in the winter (Geist, 1998),. Chaplin (1977) described dropping antlers in adult males from October, with subsequent growth of new antlers. Antlers velvet cleaning period occurred before May to June.

Bedford (1951) described in Woburn abbey double antlers cycle of Père David's deer too. Summer antlers - growth from December/January to May and dropping the September/October. Winter antlers – growth to December and the dropping after a few weeks. Double antlers occurred repeatedly in most adult males, but this phenomenon disappeared and it is rare. One from documented examples of occurrence double antler cycle (Chaplin, 1977) was occurrence in English zoo gardens. For male from Edinburgh Zoo with occurred dual antlers after transport to Calder Park.

2.1.4 Reproduction in Père David's deer

Among polygynous mammals, males differ markedly in their reproductive success. A great deal of effort has been made to understand how selective forces have shaped traits that enhance male competitiveness both before and after copulation, e.g. sperm competition (Malo et al., 2004). In polygynous sexually dimorphic species, sexual selection should be stronger in males than in females (Kruuk et al., 1999). Male reproductive success is determined by the ability of males to gain sexual access to females and by their ability to fertilize them (Malo et al., 2004). For example, Pere David's deer males are unique by swinging with penis horizontally from side to side with an upward swing at the end of the traverse (Geist, 1999).

Deer in temperate climate of northern hemisphere are seasonal breeders and show cyclic variation in testicular volume and cellular differentiation within the tubular and interstitial testis compartment (Hombach-Klonisch et al., 2004). Animals reach maturity usually during second year (Hu a Jiang, 2002). About two months before breeding season in June, males leave the herd. They rejoin a harem of females and fast during the rut. When fighting, males use antlers when growth is finished (hard antlers) or use bites

and even rise up on hind legs and box (kicks) with their front legs during antler growth (Nowak, 1999). The period of rut is very interesting because it is a time when activity of male deer is dramatically increased and reproductive/mating behaviour is easily observed. Breeding season is influenced by genetic factors, hormone levels and changes in the relative amounts of daylight and darkness in each twenty-four-hour period. This seasonal fluctuation in the timing of the melatonin increase is critical to many seasonal changes including antler cycles, winter/summer coat changes, food intake and the rut (Bubenik et al., 1990a).

Gestation length is variable and lasts between 270 and 300 days in Père David's deer (Hu and Jiang, 2002). Females have an approximately 20 day's long oestrous cycle and within a breeding season they can have multiple cycles (Nowak, 1999). One rarely two young ones are born. They are weaned in 10^{th} or 11^{th} month and adults live up to 18 years (Hu and Jiang, 2002).

2.2 Steroid hormones

Steroid hormones are crucial substances for the proper function of the body. They mediate a wide variety of vital physiological functions ranging from anti-inflammatory agents to regulating events during pregnancy. They are synthesized and secreted into the bloodstream by endocrine glands such as the adrenal cortex and the gonads (ovary and testis). Steroid hormones are all characterized by the steroid nucleus which is composed of three to six member rings and one to five member rings (Kauffman, 1999). Steroid hormones can be grouped into five groups by the receptors to which they bind: glucocorticoids, mineralocorticoids, androgens, oestrogens and progestogens. Vitamin D derivatives create sixth closely related group of hormones with homologous receptors. They have some of the characteristics of true steroids as receptor ligands (Funder et al., 1987).

Androgens are a group of chemically related sex steroid hormones. In humans and other vertebrates, androgens are made primarily in the male testes, female ovaries, and adrenal glands. Like all steroid hormones, androgens produce effects by docking with

receptors on the cell's membrane surface or inside the cell in the liquid cytoplasm (Cato et al., 2002).

Oestrogens are one of the two types of female sex hormones. They are secreted mainly by the ovaries and in smaller amounts by the adrenal glands. Functioning similarly to androgens, the oestrogens promote the development of the primary and secondary female sex characteristics. Estradiol is the most potent of the oestrogens. Progestogens are the other type of female sex hormones and they are named for their role in maintaining pregnancy. The most important of them is progesterone. Oestrogens and progestogens are secreted cyclically during oestrous/menstruation cycle (Kluger, 2015).

2.2.1 Testosterone

Testosterone is connected to sperm production (Malo et al., 2009). In cervid species, it also participates to the formation of pedicle and controls the growth of antlers. As in other species, it manages the development of secondary sexual characteristics (Li et al., 2003). In seasonally reproducing species, there is a large seasonal variability in testosterone levels. During the spring, low levels of testosterone are common and then during summer, the levels increases. In the winter, there is a rapid decrease in testosterone levels (Lincoln, 1972a).

Development of antlers mass is associated with continuous increase in testosterone concentrations in many cervid species (Bartoš et al., 2012). According to the studies of Bartoš et al. (2012), Gaspar-López et al. (2010), Malo et al. (2009), Suttie et al. (1995) and Suttie et al. (1984), testosterone levels rise during antlers growth. At first, testosterone stimulates the growth of antlers and after exceeding value, the growth of antlers is stopped through mineralization. After this, males of cervid species are cleaning velvet and then rut season begins. The growth of new antlers is connected with short-term testosterone pulses. Dominant individuals have earlier and higher increases of testosterone levels that enables to have more time for antler growth and thus larger antlers in next season (Bubenik, 1990). Cleaning of velvet arrives in a period when the testosterone levels increase seasonally (Lincoln, 1992). The fact

that the beginning of antler growth is accompanied by low testosterone levels was confirmed by the study conducted to castrate males. Males after castration were able to achieve the same size of antlers as the males that were not castrated. However, antlers remained in velvet (Price et al., 2004, Li et al., 2003).

Testosterone has a direct effect on the aggressiveness of cervid males. According to Lincoln et al. (1972b), the males are less aggressive when they are castrated. Also, when castrates were implanted with testosterone, they developed all aspects of rutting behaviour within a few weeks and showed conspicuous aggressiveness to other stags.

2.3 Determination of hormones

For determination of hormones, several methods of samples collection can be used. These methods can be divided into two basic groups. These groups are invasive and non-invasive methods and their separation is based on effect that each group of methods have on physical state of studied animal. For different samples, specific enzyme immunoassays were developed, from which it is possible to get metabolite measures (Kobelt et al., 2003).

2.3.1 Invasive and non-invasive methods

Invasive methods cover process of sample collection when physical interference of animal bodies is penetrated, such as for example cut, puncture and so on. The blood collection is the most frequent invasive method of samples collection with subsequent analysis of hormone‘s concentration from blood plasma. Advantage of this method is the instantaneous direct measurement of hormones in blood, without influence of their conversions (Kobelt et al., 2003).

However in wild animals or in some zoological gardens, blood taking can be dangerous or impossible (Möstl and Palme, 2002). In these cases, non-invasive methods are used. Among non-invasive methods, such processes during which physical interference to the bodies does not take place belong. With the usage of these methods,

hormones are not determined from the blood plasma, but from alternative sources as saliva (Greenwood and Shutt, 1992), urine (Bamberg et al., 2001), faeces (Pereira et al., 2005), milk (Rabiee et al., 2002a), feathers or coat (Bortolotti et al., 2008).

Nevertheless, also these media have some limitations. For analysis of milk or urine, it is necessary to manipulate with animals for samples collection and application of these methods is limited in usage only in some individuals, e.g. milk can be obtained just from lactating females. Urine collections in free living animals, where experimenter has not suitable conditions to obtained samples, is nearly impossible. Similar limitations exist for collection of saliva, where the size and dangerousness of animal can be another difficulty. Therefore, faecal samples seem to be the most optimal of all mentioned media for analyses of hormone levels (Möstl and Palme, 2002). During faecal analyses, metabolites are extracted from faecal samples that were freshly collected or preserved by freezing shortly after defaecation, lyophilised or cured in alcohol (Palme, 2005). Usage gives both, however, freshly collected samples are more suitable because of more simple manipulation (Möstl and Palme, 2002).

Advantage and preferred application of non-invasive instead invasive collection methods is not just in the reality that the design of collection is simpler, repeatable and without necessary equipment or handling facilities (Palme, 2005). It is also because after application of these methods, levels of measured hormones will not be influenced. In addition, with the usage of these methods, it is possible to long-term monitor the animal without interruption of its physical state and display of detrimental effects of such monitoring (Hirschenhauser et al., 2005).

2.3.2 Enzyme Immunoassay and Enzyme-Linked Immunosorbent Assay

Enzyme immunoassay (EIA) and enzyme-linked immunosorbent assay (ELISA) are both widely used as diagnostic tools in medicine and as quality control measures in various industries. They are also used as analytical tools in biomedical research for the detection and quantification of specific antigens or antibodies in a given samples (Stephanie et al., 2013).

EIA and ELISA are derived from the radioimmunoassay (RIA) (Stephanie et al., 2013). RIA is an in vitro assay that measures the presence of an antigen with very high sensitivity. Basically any biological substance for which a specific antibody exists can be measured, even in minute concentrations. RIA has been the first immunoassay technique developed to analyse nanomolar and picomolar concentrations of hormones in biological fluids. The target antigen is labelled radioactively and bound to its specific antibodies. A limited and known amount of the specific antibody has to be added. Because of the safety concern regarding its use of radioactivity, RIA assays were modified by replacing the radioisotope with an enzyme, thus creating the modern EIA and ELISA methods (Patron et al., 1987).

EIA/ELISA uses the basic immunology concept of an antigen binding to its specific antibody, which allows detection of very small quantities of antigens such as proteins, peptides, hormones, or antibody in a fluid sample. EIA and ELISA utilize enzyme-labelled antigens and antibodies to detect the biological molecules. The most commonly used enzymes are alkaline phosphatase and glucose oxidase. The antigen in fluid phase is immobilized, usually in 96 well microtiter plates. The antigen is allowed to bind to a specific antibody, which is itself subsequently detected by a secondary enzyme-coupled antibody. A chromogenic substrate for the enzyme yields a visible colour change or fluorescence indicating the presence of antigen. Quantitative or qualitative measures can be assessed based on such colorimetric reading. Fluorogenic substrates have higher sensitivity and can accurately measure levels of antigen concentrations in sample (Patron et al., 1987; Stephanie et al., 2013).

The key step in the ELISA assay is the direct or indirect detection of antigen by adhering or immobilizing the antigen or antigen-specific capture antibody, respectively, directly onto the well surface. For sensitive and robust measurements, the antigen can be specifically selected out from a sample of mixed antigens via a “capture” antibody. The antigen is thus “sandwiched” between such capture antibody and a detection antibody. If the antigen to be measured is small in size or has only one epitope for antibody binding, a competitive method is used. In this method either the antigen is labelled and competes for the unlabelled antigen/antibody complex formation or the antibody is labelled and competes for the bound antigen and antigen in the

sample. Each of these modified techniques of ELISA can be used for a qualitative and quantitative purpose (Stephanie et al., 2013). The whole process is described in the Figure 1.

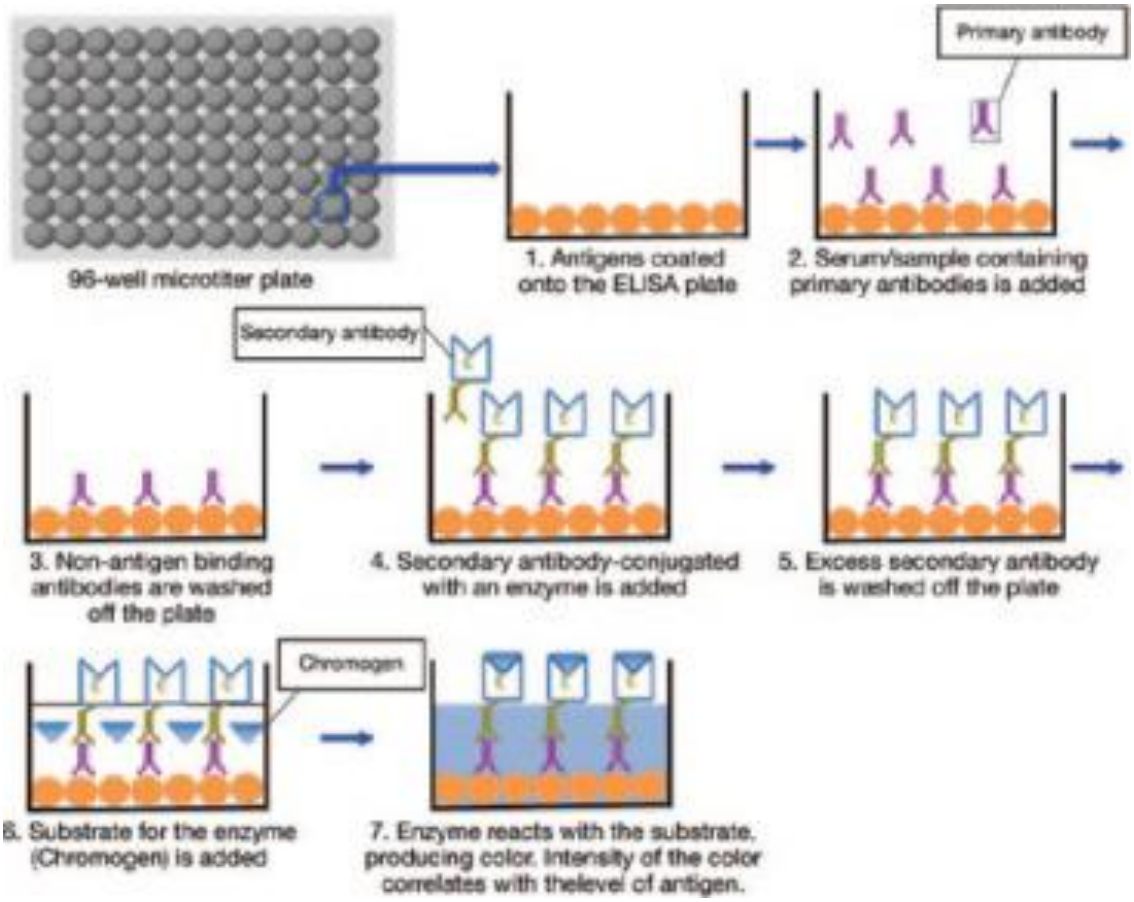


Figure 1: Enzyme-linked immunosorbent assay (ELISA) technique used to detect an antigen in a given sample. The antigen (in liquid phase) is added to the wells, where it adheres to the walls. Primary antibody binds specifically to the antigen. An enzyme-linked secondary antibody is added that reacts with a chromogen, producing a colour change to quantitatively or qualitatively detect the antigen) (Stephanie et al., 2013).

2.3.3 Types of ELISA

There are three basic types of ELISA method: direct, indirect and sandwich. All of them can be used as competitive or inhibition method of ELISA. The key event

of competitive ELISA is the process of competitive reaction between the sample antigen and antigen bound to the wells of a microtiter plate with the primary antibody. The main advantage of competition ELISA is its high sensitivity to compositional differences in complex antigen mixtures, even when the specific detecting antibody is present in relatively small amounts (Dobrovolskaia et al., 2006). The advantage of ELISA method is that for one sample analysis a combination of more types of ELISA can be used (Crowther, 2009; Straková, 2011).

Direct method of ELISA

Direct method of ELISA, which scheme is available in Figure 2, is the most simple of all ELISA methods. The process is following (Crowther, 2009; Straková, 2011):

1) Antigen is dissolved in buffer (carbonate, bicarbonate, phosphate buffer). The key of this step is that the buffer does not content any proteins that could compete during binding to the solid phase. Antigens are the main proteins that will be passively bound to the solid phase throughout incubation. The time of incubation and temperature are not necessarily controlled but the standardization is recommended. The most common temperature for incubation is 37 °C.

2) After incubations, first separation step takes place. Microtiter plates are washed by the buffer which is very important step.

3) The addition of specific antibodies follows. The antibodies are dissolved in buffer which contains other substances – blocking agents that prevent passive adsoption of protein. However, still they allow immunological binding. During incubation time, the antibodies are binding to the antigen.

4) Second washing by buffer.

5) The next step is characterized by addition of substrate or combination of substrate and chromogenic substance that will be suitable for enzyme bound to antibody. The goal is to detect the colour reaction of enzyme activity. The reaction continues for some time and then it is stopped e.g. by change of pH or by addition of inhibition substance.

6) The change of colour is quantified by spectrophotometer.

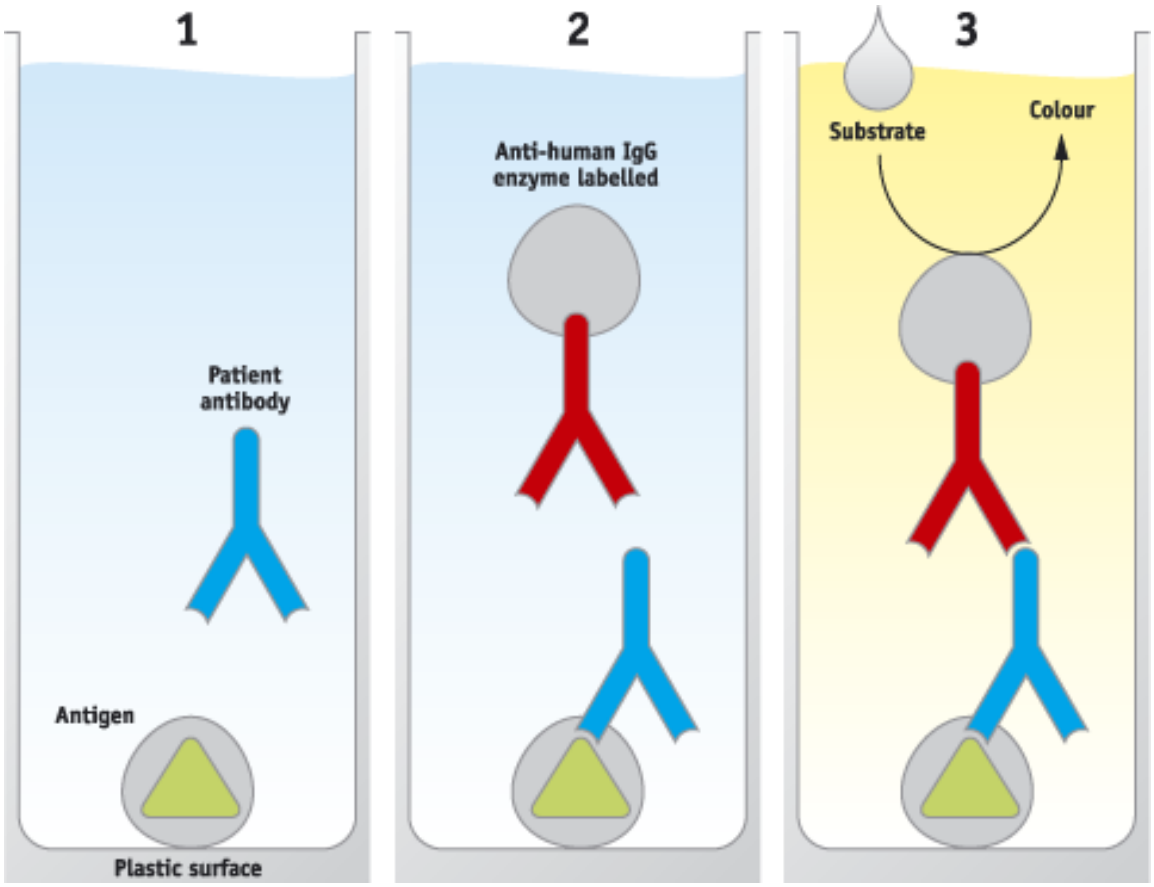


Figure 2: Scheme of direct method of ELISA (http://www.wieslab.se)

Indirect method of ELISA

This method of ELISA is very similar to the direct method of ELISA. However, it is characterized by the addition of non-marked antibodies (detection antibodies) that are dissolved by buffer. Then as in the direct method, incubation and washing of non-bound antibodies takes place. In the next step, conjugate (secondary antibodies marked by enzyme) is added. Then again, incubation and washing are realized. Finally, substrate is added to bind with antibody and create a colour reaction that is stopped after some time. Again, the change of colour is quantified by spectrophotometer (Crowther, 2009).

The common characteristic of direct and indirect methods of ELISA is that the antigen is bound to solid phase. The difference is that the indirect method uses detection antibodies that are not marked by enzyme and that serve as a target for other (secondary) antibodies that are already marked by enzyme (Straková, 2011). The

scheme showing the difference between direct and indirect ELISA methods is available in Figure 3.

This ELISA method has some advantages and disadvantages. The main advantage of the indirect method is that it is possible to test more antisera to one antigen with the usage of just one enzyme marked conjugate. This can be used for diagnostic purposes, especially when high number of samples is tested (Crowther, 2009; Straková, 2011). On the other hand, the main disadvantage of indirect ELISA method is that the antigen immobilization is not specific. However, this problem can be solved by testing more sera to get reliable results (Stephanie et al., 2013).

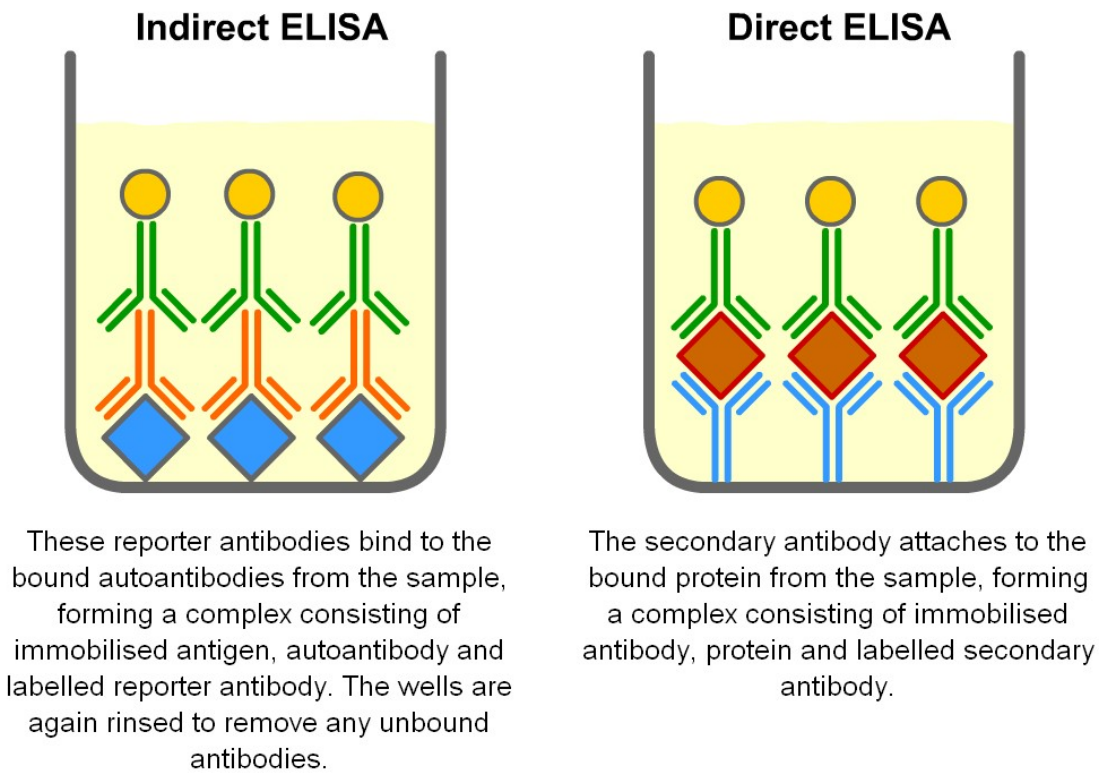


Figure 3: Schematic description of the difference between direct and indirect ELISA methods (http://www.chemgapedia.de)

"Sandwich" method of ELISA

Two types of the "sandwich" method of ELISA can be distinguished – direct and indirect.

The process of the direct "sandwich" method of ELISA is following. The antibodies are passively bound to the solid phase in the first step. These antibodies then bound the antigens that are dissolved in blocking buffer to prevent non-specific binding. Blocking buffer should not content any antigens that could be potentially bound to antibodies. After incubation a washing, stable antigen-antibody complex should be created. Bound antigen should be then detected by addition of enzyme marked specific antibody with which it creates complex that is again incubated in buffer. This second antibody can be of the same kind as the first one that was used. After incubation and washing, substrate is added and the reaction takes place sometime until it stops. The results are detected by spectrophotometer (Crowther, 2009; Straková, 2011; Canady et al., 2013).

First steps of in-direct "sandwich" method of ELISA are very similar. The antibody is bound to the solid phase and then the antigen is bound on the antibody. In the next step, detection antibody that is not marked by enzyme is added. After incubation and washing, this detection antibody is detected by itself by addition of enzyme conjugate. Following steps are the same as in the previous type. The schematic comparison of "sandwich" method with both previous simple ELISA methods – direct and indirect is available in Figure 4.

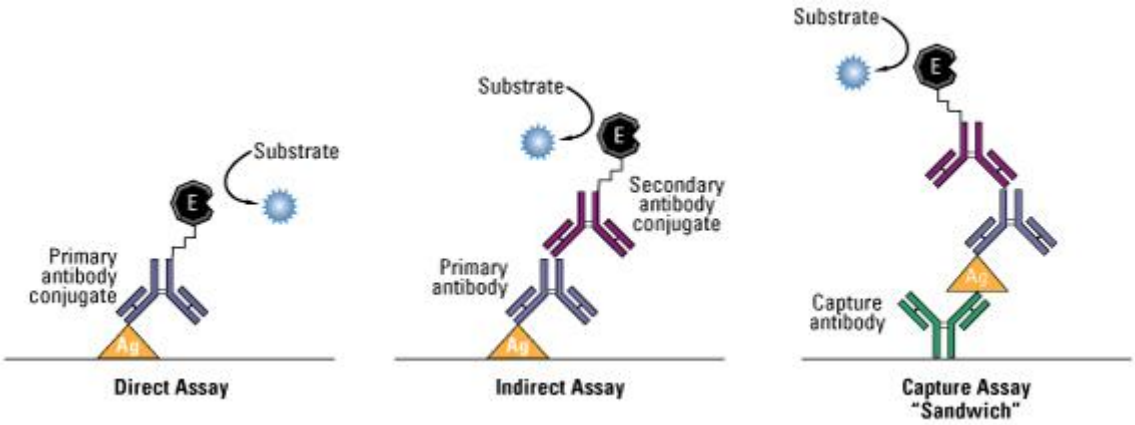


Figure 4: The schematic comparison among all three methods of ELISA – direct, indirect and "sandwich" method (https://www.thermofisher.com)

3. Aims of the Thesis

The objectives of this study were:

1. To review the methods for determination of hormones from faecal samples,
2. To evaluate seasonal endocrinology dynamics of antler cycle in Père David's deer through concentrations of faecal metabolites of androgens,
3. To compare the effects of seasonal and antler cycle to hormonal changes in males of Père David‘s deer and potential effects of position in hierarchy (rank) and social environment (only male group vs. males and females group) .

4. Hypothesis

We hypothesized that:

1. Males will have different concentrations of faecal metabolites of testosterone and epiandrosterone different during stages of antler growth including reproduction period, with the highest levels before cleaning of velvet and casting.
2. Rank of animal in males group will influence levels of faecal metabolites of testosterone and epiandrosterone.
3. Social environment will influence levels of male faecal metabolites of testosterone and epiandrosterone with the higher levels in groups where females are present.

5. Material and Methods

5.1 Literature review

For articles searching, following keywords were used: antler cycle, *Elaphurus davidianus*, Enzyme Immunoassay, epiandrosterone, metabolites of hormones, testosterone in online data bases of Web of Knowledge, Web of Science and in bibliographic database Google Scholar.

5.2 Animals included in the study

We monitored 10 adult males of Père David's deer (*Elaphurus davidianus*) further reffered as PDD from Institute of Animal Science in Prague-Uhříněves (IAS, n = 6) and five zoological gardens, namely Ostrava Zoo (n = 1), Brno Zoo (n = 1), Bratislava Zoo (n = 1), Prague Zoo (n = 1) and Chomutov Zoo (n = 1). In all males, antler development and and period of rut was monitored. Unfortunately we have not succeed to finish sample evaluation of Chomutov zoo, therefore those are not included in results.

5.2.1 Animals from the Institute of Animal Science in Prague-Uhříněves (IAS)

All 6 males were kept in approximately 1,000 m^2 large deer facility consisted from large paddock with tress and the shelter (old horse barn). The feed ration of animals consisted from meadow hay that was provided *ad libitum* and 0.5 kg of grains (mixture of oats and soya with minerals) per individual per day. Salt licks and water was provided *ad libitum*. There was no grass cover in the paddocks.

Distinguishing of individual males was done according to collars of different colours that animals wore all the time (white, yellow, red, blue, green, no collar/Bob).

The male with the no collar was the only animal that was named, its name was Bob and the name was used in legends of graphs represented by black colour.

The age was different among males and the bachelor herd showed signs of hierarchy. Two males were born in 2010 – yellow (*6. 4. 2010) and red (*14. 4. 2010). The rest of the males were born in 2009 – white (*25. 3. 2009), blue (*24. 3. 2009), green (*10. 4. 2009), black/Bob (*4. 5. 2009).

To assess dominance relationships among animals, agonistic interactions during concentrate feeding (barley groats) were observed weekly throughout the entire survey. Any approach, attack, threatening gesture, or sparring that took place between two animals and caused an apparent displacement of one individual was recorded, and the animals were categorized as ’winner’ or ’loser’. A dominance index based on win-loss scores was then calculated according to dominance index (DI), calculated as DI = D/ (D + S), where D = number of “ascertained” relationships, in which the animal was categorised as ’winner’, S = number of “ascertained” relationships, in which the animal was categorised as ’loser’ (Sambraus, 1975; Wierenga, 1990). Animals were ranked in hierarchical order according to this index. The dominance index of the highest ranking animal had the highest value.

5.2.2 Animals from the zoological gardens

In all zoological gardens, the size of the paddock was relatively comparable around 1,000 m^2 and it consisted from the shelter and free range with trees. All enclosures looked similar, they were indented and fenced by fences suitable for game. The feed rations of animals were very similar in all zoos. It was based on hay *ad libitum*, vegetable and 0.3- 0.7 kg grains (e.g. wheat, barley and oats) per individual per day (sometimes in the form of pellets for cervid species). In some zoos, straw, acorns and fruit were also provided occasionally. Salt licks and water were provided *ad libitum*. There was no grass cover in the paddocks, but some plants emerged time to time.

All males in zoological gardens were kept together with female(s) (Bratislava 2 females, Brno 6 females, Chomutov 9 females, Ostrava 6 females, Praha 2 females) and

from spring to winter also with born calves before calves were transported to other breeders. Samples were collected from only adult and dominant male in each enclosure in case there was more males (i.e. Bratislava 2 males, Chomutov 5 males, Ostrava 2 males).

5.3 Samples collection and storage

Collection of faecal samples took place from 2010 to 2012, concretely in following periods: March 2011 – June 2012 in the IAS and Ostrava Zoo, November 2010 – December 2012 in Prague Zoo, March 2011 – March 2012 in Bratislava Zoo and July 2011 – May 2012 Brno Zoo. Sampling was managed in approximately 7-9 days intervals with few exceptions when the interval was prolonged to 14 days.

Freshly voided faecal samples were collected (usually right after defecation) to sealed plastic bags that were marked by name of the animal, date (day-month-year) and place of collection. Immediately after sampling, samples were transferred to the freezer (t = -20 °C) where they were stored until transportation to laboratory processing. For transportation, special polystyrene boxes with freezer gel pack were used.

5.4 Methodological procedure

The whole processing of the samples was realized in the University of Veterinary Medicine, Vienna at the Faculty of Chemistry. For the evaluation of the samples, specific method procedure was used and it was adjusted to PDD.

All samples were removed from the freezer 30 minutes before laboratory analysis to melt. Before weighing (Figure 4), all solid material different from faeces was removed.

Figure 5: Faecal samples in sealed plastic bags and example of sample weighing (Photo by Zuzana Klapalová)

For measuring the dry matter content of the faecal samples, 50% proportion of each sample was dried at 120 °C to constant weight. The dry substance content of each sample was calculated as follows: $\alpha = C/G$, where C represents the constant weight of the sample and G means the gross weight of the sample. The index $\beta = 1/\alpha$ was used as a correction factor to transfer wet faecal sample steroid concentration into dry faecal sample steroid concentration (Li et al., 2001). The index was developed by Wasser et al. (1991), who described the technique of choice to extract faecal testosterone, estradiol and progesterone from the wet faecal samples.

5.2.1 Testosterone

As the first step for testosterone analyses, 0.5 g of wet faecal samples was placed into a test tube. Then, 4 ml of a mixture of analytically pure methanol and distilled water (dH_2O) were added to the test tube and the content was homogenized by vortexing the tube for 15 minutes. For lipid extraction, 2.5 ml of analytically pure petroleum ether were added and the test tube was again vibrated for 0.5 minute. After this, centrifugation at 1500 revolutions per minute (rpm) for 10 minutes at room temperature took place. Upper liquid layer was discarded and 2 ml of methanol layer was transferred to another tube and dried at 70 °C. For future analysis, dried samples were redissolved with 2 ml of phosphate buffer solution (0.1 M, pH = 7.0) (Figure 6) from the last samples (Li et al., 2001).

Figure 6: Redissolving of the samples by phosphate buffer solution for testosterone analyses (Photo by Zuzana Klapalová)

5.2.2 Epiandrosterone

For epiandrosterone analyses, 0.5 g of wet faecal samples was placed into a test tube. Then, 3.5 ml of analytically pure dichloromethane and 1.5 ml potassium hydroxide (0.1 M) were added to the test tube and the content was homogenized by vibration of the test tube for 3 minutes. The mixture was then centrifuged at 1500 rpm for 10 minutes. Then, the liquid layer was discarded and 3 ml of extracted dichloromethane were transferred into another tube. Dichloromethane solution was washed twice by dH_2O. After this, 1 ml of dichlormethane solution was extracted and evaporated at 45 °C. For epiandrosterone determination, dried samples were redissolved with 0.6 ml of gelatine phosphate buffer solution (0.1%) (Figure 6) from the last samples (Li et al., 2001).

Figure 7: Redissolving of the samples by phosphate buffer solution for epiandrosterone analyses (Photo by Zuzana Klapalová)

5.5 Statistical procedure

All analyses were performed using statistical software SAS System V 9.4 (SAS Inst. Inc., Cary, NC). To fit the skewed concentrations of hormone metabolites into a normal distribution, log transformation was applied. Data normality was assessed by plotting histograms and normal probability plots. Four different tests were performed (Shapiro–Wilk, Kolmogorov–Smirnov, Cramer–von Mises and Anderson–Darling). Afterwards, the transformed concentrations of testosterone and epiandrosterone metabolites were included as a dependent variable in analyses. Each was analysed separately using the Generalized Linear Mixed Model (GLMM). The Stage of antler growth or reproductive period was then used as level of explanatory variable including ’antler growth‘, ’cleaning velvet‘, ’rut‘, ’after rut‘ and ’antler casting’ further another explanatory variable Place as ‘zoo or IAS’ and finally interaction between Stage of antler growth and Place. To account for repeated measures on the same animals over the experimental period, analyses were performed with PROC MIXED, using the individual animal as a random factor. Differences between the effects were tested using the F-test. For multiple comparisons, we used the Tukey-Kramer adjustment.

For evaluation of influence of rank on concentrations of testosterone and epiandrosterone metabolites we used only animals of IAS, because it was only group where we had samples from more males and it was also only group without females. We use the Generalized Linear Mixed Model (GLMM). The rank represented by index of dominance for each animal was then used as explanatory variable. To account for repeated measures on the same animals over the experimental period, analyses were performed with PROC MIXED, using the individual animal as a random factor. Differences between the effects were tested using the F-test. For multiple comparisons, we used the Tukey-Kramer adjustment.

6. Results

According to observation recordings, no sign of double antler cycle in any of the males was monitored, either in the IAS or in the zoos.In total, 600 faecal samples were collected and processed. In zoological gardens, 240 samples were obtained, 60 samples from each zoo. In the IAS, 360 samples were taken, 60 samples from each individual male.

Individual seasonal changes in levels of androgen metabolites (according to stages of antler growth and reproductive period referred to rut) in all studied males are plotted in Graphs 1 and 2. It is possible to clearly see increases and decreases of these metabolites during specific stage of antler growth and reproductive period .

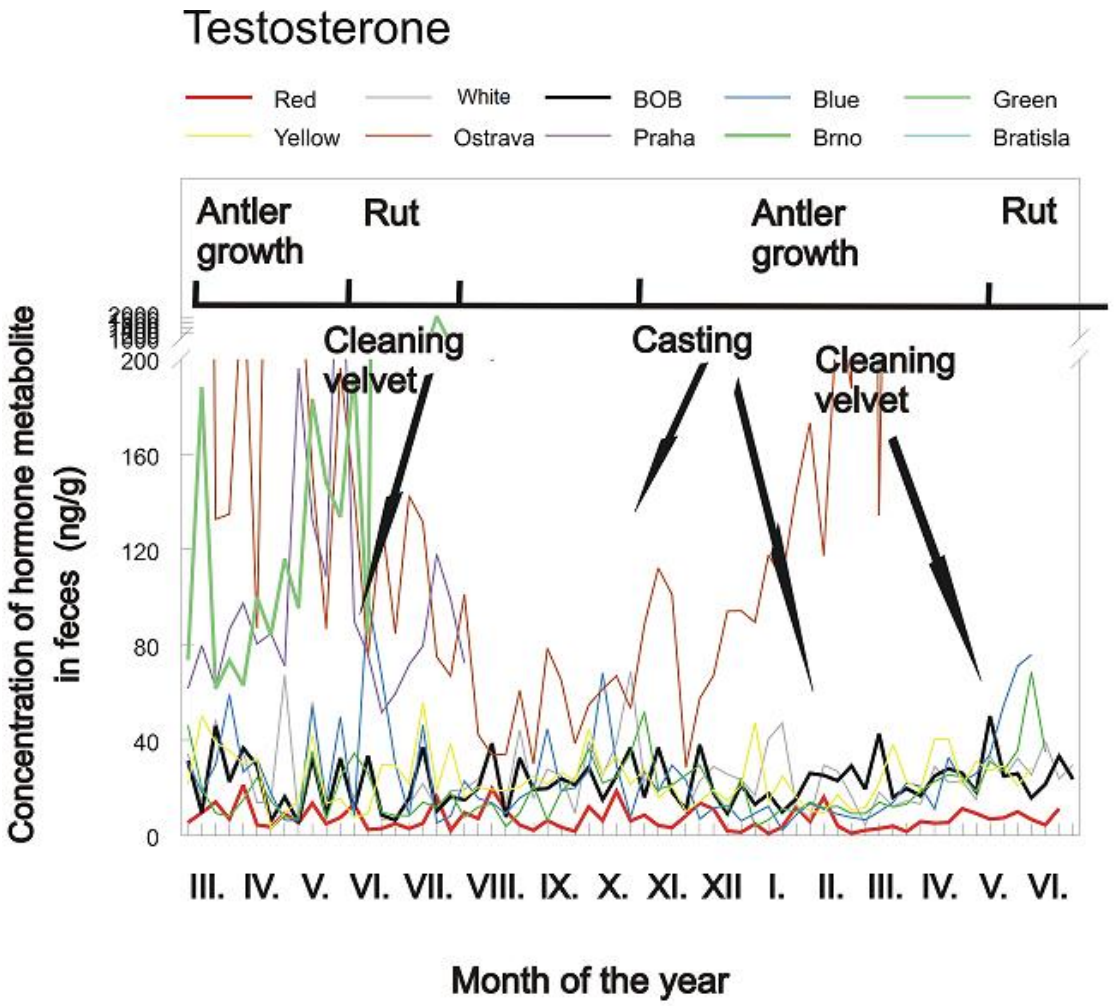
Concretely, seasonal dynamics of testosterone metabolites is plotted in Graph 1, where it is visible that the levels of testosterone metabolites noticeably rose up and decreased as the phases of the antler growth cycle or reproductive period changed.

Also, according to the Graph 1, it is visible that, in average, males from zoological gardens had higher levels of testosterone metabolites than males kept in the IAS. Also, the seasonal changes in these males were higher than in males from the IAS.

The lowest levels of testosterone metabolites were found in the male from the IAS marked by red collar who was younger and the lowest position in hierarchy. The position in hierarchy was found in group of IAS males significant for $\log_{10}$ transformed testosterone metabolites ($F_{(5,375)} = 41.5$, $P < 0.0001$) and also for $\log_{10}$ transformed epiandrosterone metabolites ($F_{(5,375)} = 2.82$, $P = 0.0162$). In both hormones higher rank represented also higher measure of faecal metabolites. Therefore, secong hypothesis was confirmed.

Rank (hierarchical order) assessed in IAS group according to Dominance index (higher the index means more dominant) was:

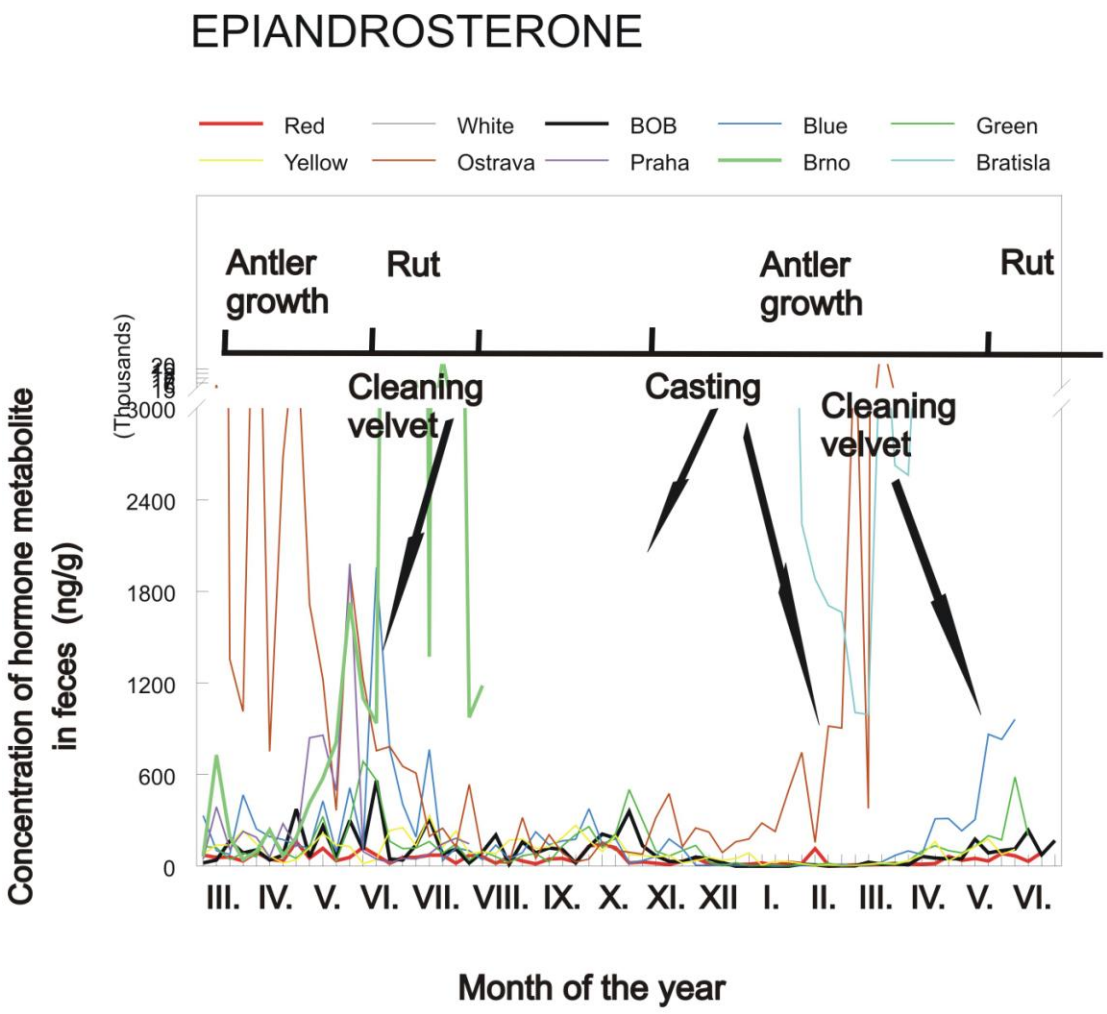
Position	Name	Dominance index
1	blue	0.71429
2	bob	0.57143
3	white	0.45857
4	yellow	0.40321
5	green	0.28571
6	red	0.14286



Graph 1: Seasonal dynamics of testosterone metabolites in all studied individuals

Similarly as with testosterone metabolites, seasonal changes (according to stages of antler growth and reproductive period) in levels of epiandrosterone metabolites are plotted in Graph 2.

Similarly to testosterone, the lowest levels of epiandrosterone metabolites were found in the male from the IAS marked by red collar.



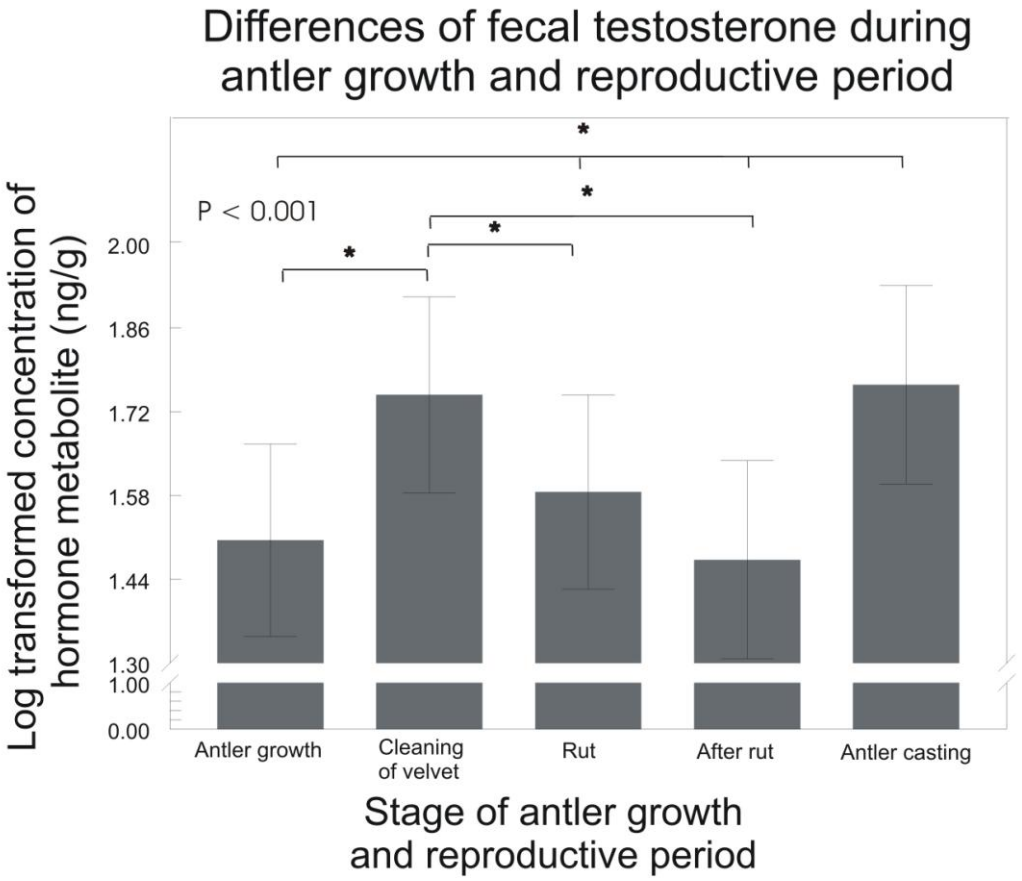
Graph 2: Seasonal dynamics of epiandrosterone metabolites in all studied individuals

Mean values of androgen levels of all individuals together in different stages of antler growth or reproductive period referred to rut are available in Graphs 3 and 4.

We proved that different stage of antler cycle or reproductive period resulted in different level of concentration of faecal metabolite of $\log_{10}$ transformed testosterone

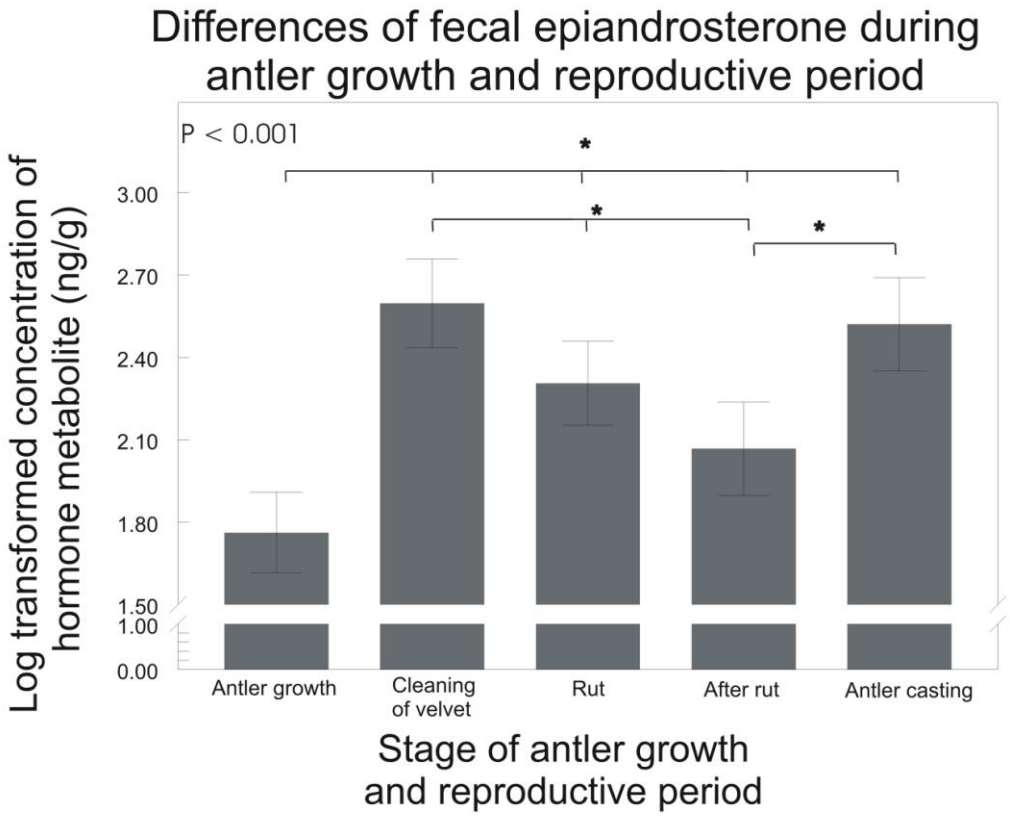
faecal metabolites ($F_{(4,489)}$ = 13.64, P < 0.0001) and log_{10} transformed epiandrosterone faecal metabolites ($F_{(4,489)}$ = 35.46, P < 0.0001).

Comparison of mean levels of testosterone faecal metabolites of all individuals together in different phases of antler growth or reproductive period is available in Graph 3. In Graph 4, the same is presented for epiandrosterone faecal metabolite levels.



Graph 3: Log_{10} transformed testosterone metabolite levels of all individuals together in different phases of antler cycle or reproductive period. Asterisk marks significant differences

The highest concentrations of faecal metabolites of hormone testosterone were found during velvet cleaning and antler casting period and the lowest during antler growth and after rut (reproduction) period. We compared individual phases to each other on the basis of P < 0.001.



Graph 4: Log_{10} transformed epiandrosterone metabolite levels of all individuals together in different phases of antler cycle or reproductive period. Asterisk marks significant differences

The highest concentrations of faecal metabolites of hormone epiandrosterone were detected during cleaning of velvet and antler casting and the lowest during antler growth period. As in previous part we compared individual phases to each other on the basis of $P < 0.001$. Regarding this comparison, differences in the concentrations of these specific stages are well defined in all cases. Together above mentioned indicates, that also first hypothesis was confirmed.

Place (zoo or IAS) was also found significant for both faecal hormone metabolites, for log10 transformed testosterone ($F(4,489) = 35.46$, $P < 0.0001$) and log10 transformed epiandrosterone ($F(4,489) = 35.46$, $P < 0.0001$). Males in zoos had higher levels of log10 transformed testosterone (LSMeans ± SE, 2.114 ± 0.106 vs. 1.262 ± 0.084) and higher levels of log10 transformed epiandrosterone (LSMeans ± SE, 2.56

± 0.149 vs. 1.992 ± 0.115). The interaction between Stage of antler growth and Place was also significant for log10 transformed testosterone ($F(4,485) = 3.46$, $P = 0.0084$) and log10 transformed epiandrosterone ($F(4,485) = 5.39$, $P = 0.0003$) being in all stages and faecal metabolites higher for males in zoos. Therefore, we confirmed also the third hypothesis.

Discussion

Even that concentrated feed was provided to all tested males, no sign of double antler cycle was monitored during research period in all tested males on contrary of other years where adult male in Prague and Ostrava zoo had double antler cycle. Therefore, we were not able to include this effect is hormone level evaluation. It is possible that the amount of concentrated feed or some other specific situation in male was excluding males to grow the second set of antlers (Chaplin, 1977; Naish, 2011). However, Chaplin (1977) declared that the double antler cycle in Père David's deer could be also connected with the environmental triggers. This would suggest that the environmental condition in the Czech Republic and Slovak Republic during faeces sampling was not suitable for double antler cycle (Chaplin, 1977). However, there is historical evidence that even in the Czech Republic, double antler cycle was observed in Père David's deer (Jaczewski, 1983). Nevertheless, the results correspond with the fact that double antler cycle is considered less frequent in some years (Bedford, 1951).

From the results we confirmed that the levels of both tested hormones faecal metabolites, testosterone and epiandrosterone, were changing throughout the year in tested Père David's deer males, which corresponds with many studies in other cervid species (Suttie et al., 1984; Suttie et al., 1995; Malo et al., 2009); Gaspar-López et al., 2010; Bartoš et al., 2012).

The lower levels of both tested hormones metabolites were found in males from IAS (only male group) . Because the hormonal levels are also influenced by position in hierarchy (rank order) (Bubenik, 1990; McCullough, 2008) and dominant males may have usually higher levels of testosterone (McCullough, 2008), those high ranking males had higher levels of hormone metabolites than lower ranking ones.. It corresponds also with the fact that lowest ranking male was the youngest male of the males herd.

The levels of testosterone and epiandrosterone metabolites were also connected with antler growth, being higher in males that were kept in zoological gardens than in males kept in the IAS. This could be caused by the presence of the females

in zoological gardens' enclosures together with males because of the stimulated production of testosterone in males. Nevertheless, in all zoos there was just one adult male in the herd which means that it was also the dominant male. Because the dominant males are known to have higher levels of testosterone than others (McCullough, 2008), it is logical that all males from the zoos had higher levels of testosterone than the majority of all males from the IAS which were also under certain stress from mutual agonistic encounters.

The average concentrations of testosterone metabolites that were calculated from the data from all individuals together separately for phase of antlers growth and reproductive periods showed similar pattern as the epiandrosterone metabolites levels. In both hormones, the lowest levels were detected during antler growth and after rut periods. Moderate concentrations were found during rut season in both cases and the highest average levels of testosterone and epiandrosterone were measured in during periods of velvet cleaning and before antler casting. All these results are very similar to the results of other authors for other species of deer (Suttie et al., 1984; Suttie et al., 1995; Malo et al., 2009); Gaspar-López et al., 2010; Bartoš et al., 2012).

Obtained results confirmed our hypotheses, i.e. that concentrations of faecal testosterone and epiandrosterone metabolites differ during stages of antler or reproductive cycle with the highest levels before cleaning of velvet and casting periods. Further were influenced also by social environment and rank.

Considering all the results, seasonal changes in tested hormonal levels can be described in the connection with antler cycle and reproduction state of animals. Gradual increase in concentrations of testosterone metabolites was visible during spring and summer season when animal had hard antlers, which is opposite period in comparison to other cervid species from temperate areas of northern hemisphere (Bartoš et al., 2012) except roe deer. As was described in the study of Lincoln (1992), the highest levels were reached in the end of this season when cleaning of velvet took place. Then, levels were lowering throughout the rut and after rut season as e.g. in the study of Asher (1989) in fallow deer (*Dama dama*). After this, casting period was represented by higher levels of hormones (Bartoš et al., 2012) and the whole cycle was going to repeat. The changes in epiandrosterone metabolites levels were similar to testosterone ones.

The situation in Père David’s deer is different than in red deer in which the levels of sex hormones are lower during the spring season, they increase before and during rut (end ofsummer and autumn) and after this, in the winter season, there is a rapid decrease (Lincoln, 1972a).

7. Conclusion

In the review part of the thesis, the methods for determination of hormone metabolites from faecal samples were described.In the experimental part of this thesis, levels of androgen hormone metabolites, testosterone and epiandrosterone, were monitored in faecal samples of Père David's deer. Samples were collected from male individuals from five zoological gardens and the Institute of Animal Science in Prague-Uhříněves. Because the individuals came from different places, it was possible to compare the levels of hormone metabolites in different social environments meaning only males group and groups with females.

It was found out that there was high seasonal dynamics in levels of testosterone and epiandrosterone metabolites in Père David's deer throughout the year according to stage of antler growth and reproduction period. In both studied hormones, seasonal changes were very similar. The highest concentrations of testosterone epiandrosterone metabolites were during period of velvet cleaning and antler casting period and the lowest during antler growth and after rut.

We confirmed that presence of females in group resulted in higher levels of testosterone and epiandrosterone faecal metabolites during whole year. In case of only male group, position in hierarchy influenced also levels of levels of testosterone and epiandrosterone faecal metabolites being higher in higher ranking animals.

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