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Noninvasive Fecal Monitoring of Hormones in Female Cheetahs

Master's Thesis

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Statutory Declaration:

I declare that I have developed and written the enclosed Master's Thesis completely by myself, and have not used sources or means without proper citation in the text.

In Prague 2016

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Abstract:

Successful breeding in captivity is essential for creating a sustainable population of cheetahs, in order to prevent their complete disappearance from the wild. Howerver, it is not easy due to several complications: (1) it is very difficult to determine if a female is in estrus; (2) female cheetahs are not always willing to mate with a selected male; (3) the conception rate and litter size is low in captive-bred cheetahs; and (4) the cub mortality rate is very high. In order to find a solution for the problems related to breeding cheetahs, it is necessary to obtain as much knowledge as possible. One of the aims of this research was to determine estradiol and progesterone metabolites excreted in faeces in cheetahs. I also tested how vocal and olfactory stimulation by male cheetah factors (voice, scent) influences the ovarian acivity in conspecific females using non-invasive method of monitoring sex steroids in faeces. In this study, 3 female cheetahs (*Acinonyx jubatus*) from the zoo Olomouc (Czech Republic) were exposed to audio and olfactory stimulation in order to determine its effect on female ovarian ativity. Fecal material from a resident male was used as the olfactory stimulus and a vocal record of an unknown cheetah male was used as the vocal stimulus. Fecal samples were collected daily for 44 days, sex steroid hormones were extracted and analysed by HPLC-MS/MS. The results showed a significant increase in estradiol concentrations in all females subjected to the stimulation, suggesting that acoustic and olfactory communication plays an important role in cheetah reproduction. Similar stimulation of a female prior to breeding might increase her willingness to mate and ensure better breeding success. Additionally, estradiol and progesterone metabolites were identified, including glucuronids, which are believed to be rarely produced by cats.

Key Words: *Acinonyx jubatus*; estradiol; progesterone; steroid metabolite; ovarian activity; stimulation; high performance liquid chromatography; mass spectrometry

CONTENTS

1. Introduction ... 1
2. Literature Review ... 2
   - 2.1. Reproduction of cheetahs ... 2
     - 2.1.1. Female reproductive system ... 2
       - 2.1.1.1. Macroscopic anatomy of the female reproductive system ... 2
       - 2.1.1.2. Microscopic anatomy of the female reproductive system ... 4
       - 2.1.1.3. Estrous cycle in felids ... 5
       - 2.1.1.4. Follicular development and ovulation ... 8
     - 2.1.2. Neuro-humoral control of reproduction ... 10
       - 2.1.2.1. Regulation of reproduction by nervous system ... 10
       - 2.1.2.2. Reproductive Hormones Origin and Action ... 11
       - 2.1.2.3. Hormonal control of ovulation ... 18
     - 2.1.3. Reproduction of cheetahs in nature ... 19
     - 2.1.4. Reproduction of cheetahs in captivity ... 23
       - 2.1.4.1. Problems affecting captive cheetah population ... 25
       - 2.1.4.2. Possible solutions to breeding problems ... 29
   - 2.2. Endocrine monitoring of ovarian activity in felids ... 30
     - 2.2.1. Fecal steroid extraction in felids ... 31
     - 2.2.2. Methods of non-invasive monitoring of sex steroids in felids ... 33
       - 2.2.2.1. Immunoassay methods ... 33
       - 2.2.2.2. Mass spectrometry ... 35
     - 2.2.3. Hormonal profiles in felids ... 39
       - 2.2.3.1. Estrus cycle ... 39
       - 2.2.3.2. Pregnancy ... 39
       - 2.2.3.3. Pseudo-pregnancy ... 40
3. Aims of the thesis ... 41
4. Material and methods ... 42
   - 4.1. Animals ... 42
   - 4.2. Fecal samples collection and stimulation of the females ... 43
   - 4.3. Fecal steroid extraction ... 43

4.4. HPLC-MS/MS analysis 44
4.4.1. Metabolic fingerprinting 45
4.4.2. Metabolic profiling 46
4.5. Statistical analysis 47
5. Results 48
5.1. Metabolic fingerprinting 48
5.2. Metabolic profiling 49
5.3. Statistical analysis 54
6. Discussion 64
7. Conclusion 68
8. References 69

LIST OF TABLES

Table 1 Female cheetahs investigated in this study 42
Table 2 Dietary plan of the studied cheetah females 42
Table 3 Dates of fecal samples selected for PCA analysis.. 46
Table 4 Identified substances of steroidal character 48
Table 5 Selected steroid substances with a connection to estradiol and progesterone metabolism... 48
Table 6 Fecal estradiol and progesterone concentrations in all studied cheetah females 49

LIST OF FIGURES

Figure 1 Biosynthetic pathway of the major gonadal steroid hormones. Simplified from Foster at al. (2001). 13
Figure 2 Hormonal changes in a female cat throughout the estrus cycle (University of Wisconsin-Madison, http://www.ansci.wisc.edu) 39
Figure 3 Elution gradient of LC method for steroid determination. Created by doc. Ing. Petr Kačer, Ph.D. (UCT Prague, 2016) 45
Figure 4 Estradiol and progesterone concentrations in fecal matter in Abiba....**Error! Bookmark not defined.**
Figure 5 Estradiol and progesterone concentrations in fecal matter in Binty.....**Error! Bookmark not defined.**
Figure 6 Estradiol and progesterone concentrations in fecal matter in Gepina..**Error! Bookmark not defined.**
Figure 7 Comparison of the fecal estradiol concentrations in all three females.**Error! Bookmark not defined.**
Figure 8 Separation of clusters before (red dots) and after (blue dots) stimulation by PCA analysis 54
Figure 9 Mean ±SE estradiol concentrations in faeces of all cheetah females during individual experimental phases (** P ≤ 0.01; **** P ≤ 0.0001) 56
Figure 10 Differences among the fecal estradiol concentrations in the individual females during the first week after stimulation (* P ≤ 0.05, ** P ≤ 0.01)... 57
Figure 11 Differences among the fecal estradiol concentrations in the individual females during the second week after stimulation (** P ≤ 0.01) 58
Figure 12 Differences among the fecal estradiol concentrations in the individual females during the third week after stimulation (* P < 0.05) 59
Figure 13 Mean ±SE progesterone concentrations in faeces of all cheetah females during individual experimental phases (P > 0.05) 60
Figure 14 Differences among the fecal progesterone concentrations in the individual females during the first week after the stimulation (* P < 0.05, ** P ≤ 0.01) 61
Figure 15 Differences among the fecal progesterone concentrations in the individual females during the second week after the stimulation (** P ≤ 0.01).. 60

Figure 16 Differences among the fecal progesterone concentrations in the individual females during the third week after the stimulation (P > 0.05) 63

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
CG	Chorinonic gonadotropin
CL	Corpus luteum
d.m.	Dry matter
E1	Estrone
E2	Estradiol
E3	Estriol
eCG	Equine chorionic gonadotropin
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
FIA	Fluoroimmunoassay
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
hCG	Huan chorionic gonadotropin
HPLC	High performance liquid chromatography
IUCN	Union for Conservation of Nature and Natural Resources
LC	Liquid chromatography
LH	Luteinizing hormone
m/z	Mass to charge
MHC	Major histocompatibility complex
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
PCA	Principal component analysis
Pers. Comm.	Personal communication
RIA	Radioimmunoassay
SE	Standard error

1. Introduction

According to historical evidence, cheetahs used to live across almost whole African continent excepting the Congo Basin rainforest. They could have been found also in Asia, ranging from the Arabian Peninsula to Eastern India. These days, cheetahs have disappeared from their original Asian range. The only exception is a small, isolated population of approximately 70 - 110 individuals in the central Iran. Regarding to the African continent, more than 77 % of cheetah original distribution is now reduced just to several smaller isolated populations inhabiting Eastern and Southern Africa (Durant, 2000; Hunter, 2014).

The reasons why cheetahs live at very low density comparing to other carnivore populations are uncertain. In general, number of carnivores depends on the size of their prey populations (Bertram, 1975 in Sinclair and Arcese, 1995). But this line of reasoning cannot be applied in the case of cheetahs. Even though cheetahs are characterized by extremely large litters in comparison with other felids (Sunquist and Sunquist, 2002), there exist several factors that keep the cheetah population size at low level. Those are also the reason why cheetah can be found on The IUCN Red List of Threatened Species listed as Vulnerable, while two of its subspecies – *Acinonyx jubatus spp. heckii* (Northwest African Cheetah, Saharan Cheetah) and *Acinonyx jubatus spp. venaticus* (Asiatic Cheetah, Iranian Cheetah) – are even classified as Critically Endangered (Durant et al., 2008).

Situation in captivity is even worse – relatively high infant mortality is accompanied by low numbers of successful breedings (e.g. Marker and O'Brien, 1989). Due to several captive breeding programs that offer future possibility of reintroduction of these animals to protected areas in the wild, reproduction of cheetahs has been a frequently discussed topic for several years. The reasons for breeding failure are still not completely clear. Essential basis for successful breeding in captivity is detailed knowledge of animals' reproductive biology, including behavioural and physiological aspects. Apparent infertility of captive cheetah females has been investigated by several methods, some of them are presented here in corresponding chapter. Tracking

of fecal estradiol, progesterone and their metabolites gives us an opportunity to study and understand cheetahs' reproductive characteristics, such as estrus cycle, ovulation, and pregnancy, and provides us with information that is essential for further research and conservation efforts (Wasse et al., 1991). Acoustic and olfactory communication is an importnat part of wild cheetahs' mating rituals (Caro, 1994) and possibly plays an important role in female stimulation (Brown et al., 1996; Beekman et al., 1997; Bircher and Noble, 1997; Wielebnowski and Brown, 1998). Simmilar stimulation might improve female's reproductive performance in captivity.

2. Literature Review

2.1. Reproduction of cheetahs

2.1.1. Female reproductive system

The female reproductive system of mammals has several major functions: beside production of reproductive hormones and female gametes (eggs), it serves as a transport canal for oocyte(s) and provides a meeting place of the female and male gametes; it enabels transportation of the zygote to the uterus, which serves as a site for fetal development; and also provides a birth canal during parturition (Kwan, Tufts University).

2.1.1.1. Macroscopic anatomy of the female reproductive system

The ovary is an internal paired organ with firm consistence and an ovoid shape (Červený, 2011), although an active ovary in polytocous (litter-breeding) animals resembles a bunch of grapes (Pineda and Dooley, 2003). Also the size and the shape of ovaries vary throughout the reproductive cycle, mainly in connection with follicles maturation and formation of corpus luteum (CL). Ovaries are located in abdominal cavity and positioned caudally from the kidneys, very close to the tips of uterine horns. They are attached to the abdominal wall by a suspensory ligament – *mesovarium*. Other supportive ligament – *mesosalphinx* – covers the oviducts and the ovaries and together with mesovarium forms an ovarian bursa – a sack protecting the ovaries (Červený, 2011). Ovaries have two major functions – an endocrine function (producing

hormones) and a gametogonic function (producing gametes) (Pineda and Dooley, 2003).

The oviduct, also know as *tuba uterina* or Fallopian tube (Kühnel, 2003), is a paired narrow, flexible, muscular tube derived from the Müllerian ducts (El-Mowafi Diamond, 2012; Kwan, Tufts University). Its role is to capture the oocyte after follicle rupturing and transport it into the uterus (Červený, 2011). It also ensures favourable environment for capacitation of sperms and fertilization and provides nutrition for fertilized egg (Kwan, Tufts University). The oviduct is located between ovary and uterus on the edge of the *mesosalpinx* – a broad ligament supporting the oviduct (Červený, 2011) (Sebastiani, a další, 2005). In the direction from the ovary to the uterus Fallopian tube is composed of four parts: it begins with *infundibulum* – a wide funnel with a small finger-like projections called *fimbriae* along the edge which help to capture the ovulated oocyte; next part is *ampulla* – expanded and only a little undulated section – in this part fertilization usually takes place; further part is a narrowed undulated section called *isthmus*; finally the tube ends with an opening called *ostium* leading into the uterine horn (Červený, 2011; Kwan, Tufts University).

The uterus is a hollow organ located in abdominal and partially pelvic cavity (Červený, 2011). In cheetah it is binocurate – it has two horns supported by a ligament called *mesometrium* (Brown, 2011). Uterus is composed of uterine body that is partially divided by a spetum, uterine horns and a cervix representing a barrier between uterine cavity and vagina. Lumen of the uterine cervix is usually closed by numerous skin folds and thick mucus protecting the uterus against infections (Červený, 2011).

Terminating part of the reproductive tract are **vagina** and external sexual organs – **vulva and clitoris** (Červený, 2011). Vagina is a fibromuscular tube with many folds on mucosa layer (Kwan, Tufts University). It extends from the cervix to the urethra orifice. The thin wall of the vagina is very flexible and can be extended both lengthwise and widthwise, especially during parturition (Červený, 2011). The lips of the vulva are located right below the anus (Brown, 2011). The surface of the labia is finely

covered with hair and containing many nerve endings, sebaceous and scent glands (Červený, 2011).

2.1.1.2. Microscopic anatomy of the female reproductive system

Ovaries are composed of two layers – external cortex and internal medulla. The surface of the ovary is covered by surface epithelium, also called the germinal epithelium, composed by a thin layer of cubodial cells (Kwan, Tufts University). These are sometimes replaced by columnar cells (Kühnel, 2003). Although the name suggests so, the germinal epithelium does not participate in any way in the production of germ cells. The *tunica albuginea*, a layer made up of connective tissue, is right below the surface layer (Kwan, Tufts University). This layer is composed by cells and fibers and does not contain any follicles (Kühnel, 2003). A layer called the (cortical) *stroma* lies underneath the *tunica albuginea* (Kwan, Tufts University). This layer, also called *zona parenchymatosa*, is composed by connective tissue cells, myofibroblasts and interstitial gland cells. These cells of epithelioid character have a complex honeycomb structure. Small lipid driblets are stored in these cells, hence they serve as a commencing material for the androgens biosynthesis. The *zona parenchymatosa* is the only zone where follicles can be found. Oocytes containing large nucleus and conspicuous nucleolus are surrounded by an epithelium made of flattened follicular cells. Inner medulla is composed of connective tissue, muscle cells, elastic and reticular fibers, and vessels. Follicles are not present in this zone (Kühnel, 2003).

Main active cells in the ovary are granulosa and theca theca cells of the follicle. Granulosa cells are able to synthesize estrogens and progesterone from androgens under control of follicle-stimulating hormone (FSH) (Short, 1962; Hillier et al., 1977; Christin-Maitre et al., 1998). Theca cells, apart of the androgens production, serve as a structural support of the growing follicle (Young and McNeilly, 2010). Due to a lack of special aromatase enzyme that converts androgens to estrogens, theca cells are not capable of producing these steroids (Ruiz-Cortés, 2012). The layer of theca cells is highly vasculrized, enabling nourishment of the cells themselves and also communication with the pituitary axis during the ovarian cycle (Young and McNeilly,

2010). After ovulation, granulosa and theca cells are transformed into luteal cells producing progesterone (Strauss III and Barbieri, 2013).

Oviduct consists of mucosal membrane, muscle layer and serosa. Mucosa of the Fallopian tube creates system of folds. The folds close to the uterus are simple, becoming more complex towards the ovary (Kwan, Tufts University). At the end of the tube the mucosa ridges are high and branched into secondary and tertiary folds (Kühnel, 2003). The inner surface epithelium consists of two types of cells – ciliated cells that help the oocyte to travel through the tube, and peg cells with secretory function that provide nourishment for the oocyte. Their activity is hormonal dependent (Kwan). The muscle layer lies right under the mucosa. It is composed of two layers of longitudinal muscles with one layer of circular muscles between them (Kühnel, 2003). The serosa is made of connective tissue, sometimes in commbination with a simple epithelium (Kwan, Tufts University).

Uterus consists of endometrium, myometrium and perimetrium. Endometrium is a thick glandular layer covered with cylindrical epithelium. Branched tubular glands are found in the endometrial mucosa (Červený, 2011). Upper endometrial layer is designed to completely or partially degenerate after estrus or pregnancy, while basal layer provides substrate for regeneration of the surface layer (Kwan, Tufts University). Myometrium is a thick muscular layer containing elastic connective tissue, vessels and nervs. The smooth muscles are arranged in two layers – longitudinal surface muscles and circular muscles beneath them. Apparent connective tissue layer between these two muscular layers includes numerous vessels and nerves. Perimetrium covers the outer surface of the uterus (Červený, 2011).

2.1.1.3. Estrous cycle in felids

In general, estrus cycle in felids consists of four phases called proestrus, estrus, diestrus and anestrus (or interestrus) (Wildt, a další, 1998; Brown, 2011). The duration of an estrous cycle is determined as the interval between two estradiol peaks (Borque et al., 2005). Cheetahs show a significantly high variability in their ovarian cycle lenghts both among females and within individuals, ranging anywhere between 7 and 21 days (Eaton and Craig, 1973; Laurenson et al., 1992; Brown et al., 1996; Brown, 2011). The

estrus cycle lenght is one of the aspects that make cheetahs unique among other large felids, whose cycles generally take longer – approximately 20 to 30 days (lion (*Panthera leo*) Schmidt et al., 1979; snow leopard (*Panthera unica*) Schmidt et al, 1993; leopard (*Panthera pardus*) Schmidt et al., 1988; Siberian tiger (*Panthera tigris altaica*) (Seal et al., 1985; puma (*Puma concolor*) (Bonney et al., 1981; clouded leopard (*Neofelis nebulosa*) (Brown et al., 1995).

Proestrus is a phase of preparation for estrus (England and von Heimendahl, 2011) and usually lasts about one day or less in cats (Brown, 2011). Proestrus phase is connected with FSH and luteinizing hormone (LH) stimulated development of ovarian follicles causing increased production of estrogens by the ovarian granulosa cells (England, a další, 2011). This results in increased male interest (Kelly et al., 1998). Proestrus females, however, are not compliant with copulation (Brown, 2011).

Next phase is **estrus**, in cheetahs lasting approximtely 1 – 3 days according to Bertschinger and colleagues (1984), 2 – 6 days according to Brown (2011), and can be even shorter in case mating occurs (Bertschinger et al., 1984). However, Seager and Demorest (1978) observed some females being receptive for up to 14 days. This period is characterized by sexual receptivness and mating can occur (England and von Heimendahl, 2011). Follicular development continues and concentration of estradiol reaches its peak within estrus (Brown, 2011). Females exhibit certain types of estrous behaviour, such as rolling, rubbing, sniffing, vocalizing, lordosis posture and urine spraying, with increased frequency, however these displays differ among individual females (Wielebnowski and Brown, 1998). According to Bertschinger et al. (1984), rolling in the presence of a male is the most reliable sign of estrus. Copulation, rarely also non-coital stimulation by male (Brown et al., 1996), promotes GnRH release from hypothalamus resulting in an LH surge (Wildt et al., 1993). As mating occurs up to five times a day (Kitchener, 1991), LH surges after each copulation enhance follicle maturation followed by its rupturing and oocyte release (Brown, 2011).

Following phase, **diestrus**, is characterized by rebuilding of ruptured follicle into a *corpus luteum*, producing progesterone in order to establish and maintain pregnancy, if that occurs (Hunter, 2003).

Anestrus is a period when the estrous cycle is paused (England and von Heimendahl, 2011). These periods of ovarian inactivity can last for several months in cheetahs, therefore females may appear infertile unless a long-term monitoring is applied. No association with season nor synchronization of anestrus period among cheetah females were observed (Brown et al., 1996). Ovarian activity varies among and within females, as described in a study of Brown and colleagues (1996), where most of the studied females showed some ovarian activity, which was ehxibited for 25 – 80 % of time in one-year period. They also described an interesting event when one of three captive females cycled approximately 80 % of the time, while the other two females cycled only about 40 % of the time. The two young sibling females cycled only during the period of ovarian inactivity (anestrus or non-pregnant luteal phase) of the older female. This observation suggests there might exist an association between cyclicity and some kind of hierarchical ranking of the individuals, even though cheetah females are not considered as social species (e.g. Caro, 1994). Similarly, Wielebnowski and colleagues (2002) reported prolonged anestrus phase in females housed in pairs. This reproductive suppression might be a result of altered social conditions by forcing females to stay close to each other.

Female comes into anestrus also after parturition. It has been described than domestic cat (*Felis catus*) stays in this state as long as lactation persists. It is believed that the main cause of the postpartum acyclicity is supression of GnRH synthesis in hypothalamus causing suspension of follicle development for the time of lactation. That is why this state is called lactational anestrus (England and von Heimendahl, 2011). Cheetah cubs are weaned at 4 to 6 months, but they stay with their mother for additional 11 to 13 months (Kelly et al., 1998). When exactly after birth is estrus resumed in cheetahs is not known. However, in the wild, cheetah females are reported to mate and be able to conceive before the previous offspring separates, although birth occurs after the separation (Caro, 1994). When cubs are prematurely lost or intentionally removed from the mother (e.g. for hand-rearing), ovarian cyclicity is restored very soon after, possibly within one week (Schaller, 1972 in Caro, 1994; Brown et al., 1996).

In case of absence of ovulation during one estrus, next estrus appears within 12 days in average (Asa et al., 1992). This interval is known as interestrus. Estrogen concentration stays at the base level and no sexual behaviour is expressed during this period. At the end of interestrus interval there is noticeable increase of FSH and LH secretion that stimulate growth and initial development of follicles and female thus returns into a proestrus phase (England and von Heimendahl, 2011).

2.1.1.4. Follicular development and ovulation

Follicle is a complex of specialized epithelial cells surrounding an oocyte (Kwan, Tufts University). Although most follicles contain only a single oocyte (uniovular follicles), there were findings of follicles containing up to five oocytes (polyovular follicles) in rabbits, dogs, cats and other mammals (Telfer and Gosden, 1987). Oocytes are present within the ovary since embryonal development as a part of primordial follicle (Pepling and Spradling, 2001). Primordial follicles are located in the outer cortical section of the ovary, usually just underneath the *tunica albuginea*. In carnivores, these primordial follicles are usually found in clusters, most of them retained in dormant state (Kwan, Tufts University). Ovaries of juvenile female contains up to 200,000 primordial follicles (Červený, 2011). Once the primordial follicle is activated, it becomes primary follicle. *Zona pellucida* is formed on the surface of the oocyte. It has a form of glycoprotein layer of gel-like structure. As the follicle grows, the space between granulosa cells is getting filled with a liquid, eventually forming a cavity filled with the fluid – *antrum*. Since this time the follicle is termed secondary or antral. Secondary follicle then undergoes further maturation process into its final stage – the Graafian follicle. This follicle is large and filled with follicular fluid in which the oocyte floats freely. Size of mature preovulatory follicle varies according to species; in domestic cat it is about 2 mm in diameter (Kwan, Tufts University), in cheetah 4 mm and larger (Wildt et al., 1993). Ovary of mature female contains follicles in various stages of development. Growth of the follicle is triggered by FSH produced in hypophysis; estrogen production and ovulation is controlled by LH (Kwan, Tufts University).

Ovluation is a complex event that involves hormonal, neural and other processes (Hsueh et al., 1984). Löfman et al. (1989) described typical structural changes that can be observed on an isolated ovary right before the ovulation. First the shape of the follicle changes forming a pointing tip. The surface of the follicle wall is modified from smooth and apparently homogenous into a rough apex with single cells extrusions and minor perforations. This way an early stigma is formed. Microcirculation in the follicular apex is reduced which results in an avascular zone over the follicle tip (Dahm-Kähler, a další, 2006). Based on observed leakage of granulosa cells over longer time period, Löfman et al. (1989) suggested that partial digestion of follicular wall occurs several hours before ovulation. This results in a significant thinning of the follicular wall where major rupture occurs later on (Dahm-Kähler, a další, 2006). Within minutes after the LH surge apparent increase of blood flow can be detected in ovaries. Although blood is redistributed so the most pressure is located at the base of the follicle while decreasing towards the apex (Brännström, a další, 1998). Despite that, in most cases profuse bleeding can be observed together with release of granulosa cells and the oocyte (Dahm-Kähler, a další, 2006). Part of these cells remains attached to the oocyte and a portion of them can be found in the vicinity of the oocyte serving as a supportive tissue with paracrine function. After the oocyte is released, dramatic changes occur on the Graafian follicle. The remains of ruptured follicle wall are restructured into a CL, due to the yellow lutein pigment also known as yellow body (Hunter, 2003). *Corpus luteum* is formed by granulosa lutein cells and theca lutein cells (Kwan, Tufts University) and produce progesterone that is essential for estabilishment and maintenance of pregnancy (Hunter, 2003).

Ovulation in cheetahs is believed to be mating-induced. This information is based on the results of an intensive laparoscopic survey which included sixty-eight captive female cheetahs. The study revealed no fresh luteal scars in females that had no opportunity to mate (Wildt, et al., 1993). Also Bertschinger et al. (1984), judging by the absence of pronounced progesterone peaks, came to the conclusion that mating is necessary for the LH surge initiation, that is essential for inducing ovulation. However, some studies revealed the presence of lutheal scars in non-mated females, suggesting cheetahs may ovulate spontaneously in some cases (e.g., (Asa, Junge, Bircher, Noble,

Sarri, & Plotka, 1992) (Wildt, a další, 1993) (Bertschinger, Meltzer, Van Dijk, Coubrough, Soley, & Collett, 1984). This is supported by Brown's et al. (1996) findings of elevated progesterone concentration after the estradiol surge, simmilar to that induced by mating, even in cases where mating did not occur. However, this only presented in a minority of cases.

2.1.2. Neuro-humoral control of reproduction

All processes connected to sexual development and reproduction are controlled by neuro-humoral complex. These are sexual maturation and thus puberty occurrence, spermatogenesis and maturation of follicles, CL formation, function of gonads and secretion of gonadal hormones (Ganong, 1977; Konrádová et al., 2000), thus also sexual behaviour that is correlated with hormonal level in bloodstream (Wielebnowski and Brown, 1998). Also parturition and lactation are regulated by neuro-endocrine mechanisms (Ganong, 1977). Complex system of neuro-humoral control of reproduction including hypothalamus, hypophysis and gonads is termed hypothalamic-pituitary-gonadal axis (Kufe et al., 2003). It is based on a „feedback" mechanism: gonadotropin-releasing hormone produced in hypothalamus stimulates hypophysis to synthesize gonadotropins, these promote secretion of gonadal hormones, and these hormones act on specific tissues within the reproductive system and also influence back the brain to launch sexual behaviours (Ganong, 1977; England and von Heimendahl, 2011).

2.1.2.1. Regulation of reproduction by nervous system

In general, interaction between environment and brain is the basis of the control of reproductive activity (England and von Heimendahl, 2011). Major parts of brain participating in hormonal regulation of sexual activity are hypothalamus and anterior pituitary gland (hypophysis).

Hypothalamus is a superior structure for hormonal regulation – it controls all important biological processes in the body, including sexual behaviour and reproduction (Konrádová et al., 2000). It is a fairly small area located at the base of brain between the midbrain and the forebrain (Johnson, 2013). Hypothalamus acts

mostly through pituitary gland by determining which hormone is released into blood system and in what concentration (Ganong, 1977).

Pituitary gland, also hypophysis, is a small gland that has central position within glands of endocrine secretion. It is located at the base of the brain (Johnson, 2013) and it is anatomically and functionally connected to hypothalamus. Anterior lobe of the hypophysis, sometimes also termed adenohypophysis, is associated, besides others, with reproduction. It is composed of many types of glandular cells. Each cell type produces different hormone, the only exception are gonadotropic cells, that produce two hormones – follicle stimulating hormone and luteinizing hormone (Konrádová et al., 2000).

2.1.2.2. Reproductive Hormones Origin and Action

Gonadotropin-Releasing Hormone

Gonadotropin-releasing hormone (GnRH) is a key regulator of reproduction. It is a neuropeptide that is synthesized in hypothalamus (Schneider et al., 2006), however the concrete mechanism of the pulsatile release of GnRH is not perfectly clear (Strauss III and Barbieri, 2013). Secreted GnRH is transported through the hypothalamo-hypophyseal portal system into pituitary gland where it controls release of gonadotropins, which subsequently trigger secretion of sexual steroids (Lee et al., 2008).

GnRH works on the principle of positive feedback from gonadotropins – increased concentration of estradiol in blood stream during follicular phase of estrous cycle triggers intense release of GnRH in the brain causing an LH surge from the hypophysis which results in ovulation in mammals (Herbison, 2008). Also negative feedback appears to regulate GnRH synthesis - high amounts of progesterone and prolactin secreted during pregnancy cause the suppression of GnRH and gonadotropins production which in turn inhibit the development of follicles (Strauss III and Barbieri, 2013).

Gonadotropins

Gonadotropins are key hormones in growth regulation, sexual development and reproductive function (Godine et al., 1982). This group includes follicle-stimulating hormone (FSH), luteinizing hormone (LH) and placental chorionic gonadotropins (CG).

Eventhough equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) are widely used in artifitial breeding of cheetahs in order to stimulate ovarian activity and to induct ovulation (e.g. Howard et al., 1997), they are not discussed in this thesis because these hormones do not occur naturally in felid system (Bowen, 2001).

Follicle-stimulating hormone (FSH), also known as follitropin, is a glycoprotein hormone (Hearn and Gomme, 2000) secreted by gonadotropes – specific cells of the anterior pituitary gland. Synthesis and release of FSH is regulated mainly by estradiol and inhibins produced in ovaries (Strauss III and Barbieri, 2013). Main physiological function of FSH is to support development of follicles (Hearn and Gomme, 2000). Granulosa cells under the impact of FSH transform androgens to estrogens and progesterone (Hillier et al., 1977; Caruso et al., 1993).

Luteinizing hormone (LH) or lutropin is another of the main gonadotropins. Together with FSH it belongs to the family of structurally similar glycoprotein hormones. LH is synthesized and secreted by pituitary gonadotropes. Principal target of LH are gonads where it encourages the ovarian theca and stroma cells to produce androgens (Hearn and Gomme, 2000), later used for production of other steroids (Johnson, 2013). LH is predominant in the preovulatory period of ovarian cycle (Strauss III and Barbieri, 2013) playing an important role in enhancing blood flow towards the ovaries and inducing ovulation. Ruptured follicle is then under the LH control restructured into a *corpus luteum*. After ovulation, LH stimulates theca cells to increase synthesis of progesterone and prostaglandin to the exclusion of estrogen production (Hearn and Gomme, 2000).

Gonadal Steroid Hormones

Gonadal steroids or sex steroids is a class of steroid hormones synthesized by the gonads. These are estrogens (including estradiol), progestins (including progesterone), and androgens (including testosterone – primarily male hormone, thus not further discussed). They are important for regulation of many processes in the body, mainly connected to sexual maturity and reproduction (Ruiz-Cortés, 2012).

Cholesterol serves as a precursor for all steroid hormones. It is via system of complex enzymatic reactions transformed to progesterone through pregnenolone, and further to androstenedione, which is subsequently converted into testosterone and estrone. Estrogens are synthesized through further enzymatic conversion from testosterone precursor and/or estrone transformation (Senger, 2003). This conversion of estrone to estradiol and vice versa (although in favour to estrone) occurs in blood and other tissues (Rouiller, 1964).

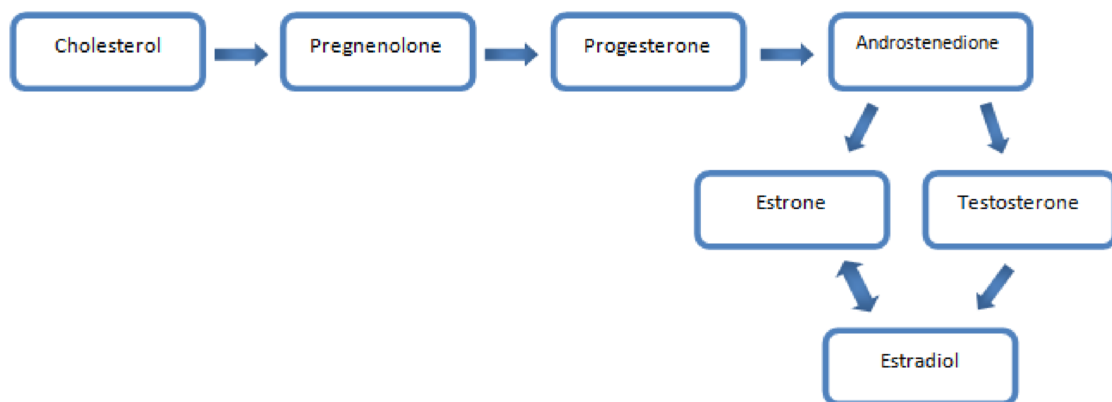

Figure 1 Biosynthetic pathway of the major gonadal steroid hormones. Simplified from Foster at al. (2001).

All steroids are metabolized (inactivated) in the liver (Senger, 2003), therefore only a little (5-10 %) free or unchanged hormone is excreted into urine or faeces (Squires, 2010). According to Cameron (unpublished data, 1961 in Rouiller (1964)), the liver is able to clear the major part of estrone during a single passage of blood in humans. Simmilar clearance effecivity of the liver was described by Rouiller (1964), illustrated by an example of high progesterone values secreted during pregnancy and low progesterone level measured in peripheral blood at the same period. Steroid

hormones undergo a series of chemical tranformations in the liver before secreted to the bile, urine or faeces. The steroidal degradation process happens in two steps – first includes chemical modification with the help of enzymes mainly by oxidative and reductive processes during which steroids are inactivated; and subsequently enzyme-mediated conjugation with glucoronic acid or sulphates, which ensures their water solubility and enables their excretion to the urine or bile salts (Squires, 2010). Part of the steroid metabolites excreted into the bile is reabsorbed by the intestines (Rouiller, 1964).

Estrogens

Estrogens are steroid hormones primarily produced by the granulosa cells of the ovarian follicle (England and von Heimendahl, 2011). Their characteristic estrane skeleton is formed by 18 carbon atoms (Ruiz-Cortés, 2012). Natural estrogens occur in body mostly in form of estrone (E1), estradiol (E2) and estriol (E3) (Khanal et al., 2006; Szymanski and Bacon, 2013). In general, estrogens are derived from androgenic precursors androstenedione and testosterone (Ruiz-Cortés, 2012) in a chemical process called aromatization (Szymanski and Bacon, 2013), or can be converted from estrone (Slaunwhite et al., 1973). Although major source of estrogens are ovaries (Falck, 1960; Wen et al., 2010), enzyme aromatase, that synthesize estrogens, can be also found in estrogen-producing cells of other tissues such as placenta, fat tissue (Simpson et al., 1994), bones (Horowitz, 1993), skin (Nelson and Bulun, 2001), testicles (Lambard et al., 2005), adrenal glands (Conley et al., 1996), and brain (Garcia-Segura et al., 1999), where estrogens can be used locally (Slaunwhite et al., 1973). However, it is not clear if feline placenta actually has the ability of producing estrogens (Braun et al., 2012).

Estrogens are able to pass through the plasma and nuclear membrane of any cell, however, they can be only retained by cells containing estrogen receptors (Nelson and Bulun, 2001). These hormones, in general, ensure normal appearence and function of female reproductive organs and throughout the estrus cycle they are responsible for majority of morphological changes of the reproductive tract. The most noticeable changes during estrus occur in uterus, where endometrial cells increase

their height and become mitotically more active, while the uterine glands intensify their production of mucus that flushes the reproductive tract. Estrogens contribute to local protection of uterus from infection by increasing amount of antibodies in local blood vessels. Increased uterine tone and contractility of myometrium during proestrus and estrus is also a result of estrogenic influence. Uterus becomes more sensitive and responsive to the influence of oxytocin and prostaglandins at this time.

Estrogens are also responsible for development of female secondary sexual characteristics such as growth and development of mammary gland, overall changes in body conformation and hair distribution. Also female sexual receptivity and willingness to mate, together with other behavioural features of estrus are controlled by these hormones (Pineda and Dooley, 2003). Estrogens produced in ovaries cause increase of blood flow and retention of water and ionts within the reproductive tract resulting in swelling of external and internal tissues (England and von Heimendahl, 2011) (Pineda and Dooley, 2003). In felids, however, the typical swelling of vulva is not as apparent as in other species, because those tissues are not so much responsive to estrogen influence (Shille and Sojka, 2010).

Estradiol primarily produced by granulosa cells and thecal tissue of the ovary (Wen et al., 2010) is the most potent of the natural estrogens and most biologically active (Geyer et al., 2000). It is most abundant estrogen during reproductive life period of a female. In the form of 17-β-estradiol it is the dominant hormone during follicular phase of estrus cycle (Pineda and Dooley, 2003). The only exception is the period of pregnancy, when estriol overcomes the level of estradiol in circulation. Also during menopause, which occures in humans and some animals after certain age (e.g. Marsh and Kasuya, 1986), the level of circulating estradiol is lower than the level of estrone (Ruiz-Cortés, 2012).

Progestogens

Progestogens are a family of steriod hormones that activate progesterone receptors (Bhattacharya et al., 2013). Typical feature for all progestogens is their basic skeleton, called pregnane skeleton, consisting of 21 carbon atoms (Ruiz-Cortés, 2012).

Progesterone is the main steroid hormone of progestogens class (Pineda and Dooley, 2003). It is produced and secreted by CL right after its formation and later on by placenta, if pregnancy is estabilished. That is the reason why progesterone is sometimes referred to as a hormone of pregnancy (Strauss III and Barbieri, 2013). However, there are speculations whether feline placenta is able to produce steroid hormones or the progesterone produced during pregnancy originates in external sources (Braun et al., 2012). According to Hunter (2003), the concentration of progesterone in bloodstream is positively correlated with the amount and size of luteal cells. This is in agreement with previous findings of Brown and colleagues (1996) that several days after estradiol surge (and formation of CL) postovulatory fecal progesterone level increased and remained elevated for 1 week or longer. That is why the highest values of progesterone are observed during diestrus phase of ovarian cycle (Ruiz-Cortés, 2012).

Progesterone is known to be involved, both directly and indirectly, in most of the processes in the body occuring during implantation and pregnancy (Strauss III and Barbieri, 2013). It facilitates fertilization by modyfying physiology of Fallopian tubes. Under the influence of progesterone spermatozoa is released from the caudal isthmus which serves as a reservoir in many species (Hunter, 2003). Right after the formation of CL and initial secretion of progesterone, the proliferation and expansion of cells in the endometrial epithelium continues, as well as the secretion of uterine glands. These increase in size and start to produce thick secretion rich in glycogen, sometimes called uterine milk, serving as a nourishment for embryo before its attachement to the uterus is complete (Pineda and Dooley, 2003; Amitrano and Tortora, 2006). Further action of progesterone causes reduction of swelling in the mucosa and tonus within the reproductive tract (Hunter, 2003) previously caused by estrogenic influence (Pineda and Dooley, 2003). Therefore the passability of the Fallopian tube increases with increasing concentration of circulating progesterone as well as the number of sperms reaching the site of fertilisation (Hunter, 2003). As Pineda and Dooley (2003) proposed, the effects of progesterone are apparent only after so called priming by estrogen, in other words after a target tissue being exposed to estrogenic influence. One of the major functions of progesterone is to prepare uterus for implantation – it suppresses

contractility of uterine wall, enhances decidualization and also has anti-inflammatory effect, which suppresses immune reaction and thus increases immunotolerance of embryo. At the same time progesterone is widely involved in production of most of the proteins synthesized by the uterus that support embryo development and implantation (Strauss III and Barbieri, 2013). Insufficient or lacking production of progesterone during pregnancy inevitably leads to abrtion, if the exogenous treatment by progesterone or similar synthetic hormone is not provided (Pineda and Dooley, 2003).

Other hormones associated with reproduction

Androgens are steroid hormones produced by theca cells of the ovary. The biosynthesis of androgens from cholesterol takes place under influence of LH. Two main androgens connected with female reproduction are androsterone and testosterone. They diffuse through cellular membrane into the granulosa cells where they serve as a substrate for estrogen synthesis. They are also believed to be a part of feedback control mechanism for LH (Pineda and Dooley, 2003).

Prostaglandins cause constriction of local blood vessels. Interruption of blood flow for longer period can lead to death of local cells – this way the wall of preovulatory follicle is weakened enabling the follicle to rupture. *Corpus luteum* degenerates at the end of the luteal phase in the same way (Johnson, 2013). Prostaglandins cause uterine contractility and are known for inducing abortions (Strauss III and Barbieri, 2013). They are also possibly involved in initiation of birth (Johnson, 2013). LH and, to some extent, FSH secretion is believed to be regulated by prostaglandins (Harms et al., 1973).

Relaxin is an insuline-like hormone that loosens pelvic ligaments enabling the pelvis to stretch during the birth process and softens the cervix, thus facilitates passage of the foetus during parturition. Relaxin possibly plays a role in lactation. In carnivores relaxin is produced mainly by CL (Johnson, 2013).

Prolactin is a protein hormone secreted by specialized cells of the adenohypophysis stimulating mammary glands to synthesize milk (Johnson, 2013).

Prolactin has also luteotrophic function, thus, together with LH, stimulates function of the CL during the luteal phase (England and von Heimendahl, 2011).

Oxytocin is a peptide hormone synthesized in thr posterior pituitary gland (Strauss III and Barbieri, 2013). It causes contractions of smooth muscles of the uterus (Johnson, 2013), and so is believed to induce parturition. It also participates in treatment of postpartum haemorrhage (Strauss III and Barbieri, 2013). Oxytocin, together with other hormones, determines maternal behaviour (England and von Heimendahl, 2011). It also stimulates the release of prolactin, thus indirectly supporting milk production in mammary glands, while directly stimulating milk ejection from those glands by causing contractions of the mammary alveoli cells (Strauss III and Barbieri, 2013). Function of the *corpus luteum* is suspended under the influence of oxytocin (Johnson, 2013).

2.1.2.3. Hormonal control of ovulation

Ovulation from the endocrine point of view is a complex event that involves cascades of reciprocally influencing hormones. The whole process is initiated by the secretion of LH under the influence of GnRH, and its binding to the LH receptors found in the highest density on granulosa and theca cells (Peng et al., 1991). LH, secreted from the piuitary gland, triggers apparent physiological changes on the ovary – blood flow increases and is redirected towards the base of the follicle (Carrell and Peterson, 2010). In cheetahs several LH surges are necessary for the follicle to rupture (Brown, 2011).

Although LH seems to be the major trigger of ovulation (Shimada et al., 2003), aslo other hormones play an important role in the ovulation process. It has been proven that FSH alone can induce ovulation in experimental conditions, however not as effectively as LH (Tsafriri et al., 1976).

Another hormone facilitating the ovulation process is progesterone. Its importance was demonstrated in a rat where ovulation was inhibited by anti-progesterone antiserum (Mori et al., 1977). In a later study, Brännström and Janson (1989) revealed that progesterone is a mediator of ovulation by supressing of

progesterone production in the ovary. It has been suggested that progesterone is involved in the breakdown of follicular wall over the apex (Tjugum et al., 1984).

After rupturing of the follicle, the oocyte is released together with a part of supportive cells that are believed to have a paracrine function – they synthesize steroids, growth factors and others, and thus support embryo development (Hunter, 2003). Cells at the place of rupturing diferentiate into luteal cells under the influence of LH (Hearn and Gomme, 2000). These play a role in the synthesis of progesterone. The concentration of progesterone secretion increases up to the level sufficient for estabilishment and maintaning potential pregnancy. This function is only temporary. *Corpus luteum* disappears sooner or later and a new one is generated after each ovulation. If fertilization takes place, CL remains on the ovary for a longer period. Even though further pregnancy maintanance is ensured by progesterone synthesis in the placenta in most mammals, feline placenta produces little or no progesterone. It appears to be produced primarily by the CL. This is supported by findings that ovariectomy or administration of luteolytic substances at any stage of gestation causes loss of the pregnancy in cats (England and von Heimendahl, 2011). Sometimes an insufficient CL is formed. Breeding time (end of breeding season when pregnancy would not be favourable due to the environmental conditions) or hormonal treatments of animals (e.g. hormonal induction of ovulation) may be the reasons for forming inadequate corpora lutea (Hunter, 2003).

2.1.3. Reproduction of cheetahs in nature

Cheetahs have very unique social and territorial patterns among felids (Sandell, 1989). While males usually live in smaller groups either ocupying a relatively small territory (these are called resident males) or territory-less males wandering over larger areas (theses are called floaters), females are solitary demanding extensive home ranges, up to more than twenty times larger than those of males (Caro, 1994). Separetly living sexes of free-ranging wild cheetahs usually meet only for the purpose of mating. Males start seeking for females after being attracted by the smell of proestrus females' urine (Kelly et al., 1998). According to Bland (1979), valeric acid may serve as the attractant. During the periods of proestrus and estrus, males try to attract

females to come and mate with them. This is done by a very unique and species-specific stutter call that males produce exclusively when stimulated by the female's urine (Caro, 1994). Females are stimulated by male presence and increased aggression among males (Herdman, 1972; N. Hulett, pers. comm. in Caro, 1994). In contrast to the observations from captivity that (e.g. Wielebnowski and Brown, 1998), Caro (1994) did not detect significantly increased occurence of any type of behaviour considered as estrus-related in the wild cheetah females.

When a male meets a female, the male starts to sniff the vegetation surrounding her. Sometimes he also attempts to sniff her perineal and vaginal area. There has been very little actual mating observed in the wild (Caro, 1994), which according to Wrogemann (1975) occurs predominantly at night. Males were reported to stay with females for a varying period of time, the shortest being 3 minutes while the longest being 2 days (Caro, 1994). Females are believed to be very picky about their partners (Bircher and Noble, 1997) and prefer to mate with unrelated individuals (Gottelli et al., 2007). In contrast to the general assumption that males are polygynous while females are monandrous (Shuster and Wade, 2003), cheetah females were found out to be more promiscuous. Mating with multiple males – resident males and also non-teritorial floaters - was proven as almost half of all litters had mixed paternity (Gottelli et al., 2007). The reason for this might be an effort to reduce infanticide by confusing males about the paternity (Wolff and Macdonald, 2004). Gestation takes 90-95 days in both wild and captive individuals (Kitchener, 1991; Caro, 1994; Brown et al., 1996). Interbirth interval in the wild cheetahs was observed to be about 20 months (Kelly et al., 1998). Cheetahs give birth to exceptionally large litters – the average number of young in a litter among most felids is 2 to 3 (Sunquist and Sunquist, 2002), while in cheetahs giving birth to six cubs is not unusual. According to some authors a cheetah‘s lair can contain even up to eight cubs at the same time (Caro et al., 1987; Caro, 1994). After birth, young weigh between 250 to 300 g (Kitchener, 1991). They are born fully furred with the characteristic spots on their greyish coat (Caro, 1994). A short time later – within 2 to 6 weeks (Marker, 2002) – a prolonged downy fur band starts to grow covering their neck and back (Caro, 1994).

Female cheetahs reach sexual maturity at 22 to 36 months (Sunquist and Sunquist, 2002). Kelly et al. (1998) state that the reproduction begins at the age of two the earliest. Caro (1994) reported the first mating in Serengeti free-ranging females occured around the age of 3. However, Wrogemann (1975) witnessed that in the wild female cheetahs are capable of having their first litter at 13 to 16 months of age. There was no limiting age for successful reproduction observed in female cheetah in the wild and, as Caro (1994) reported, even females of 15 years of age were physiologically capable of reproduction based on their folicular activity. However, the majority does not reach such an old age, as the average lifespan of cheetahs is around 6 years (Kelly et al., 1998). Laurenson et al. (1992) speculated that young females may be less fertile than older females, presupposing from interval between cubs lost and next conception, which was longer in young females. They also observed that some variation in fertility may be dependent on nutritional status of the animals, such as lowered conception rates in the dry season.

According to Caro (1994), there exists only a little evidence of breeding seasonality, however some authors disagree. Bertshinger et al. (1984) pointed to a seasonal tendency of de Wildt captive cheetahs having one main breeding season between November and February and one shorter breeding season during June and July. They remark the same seasonal pattern is noticeable also in the wild, even though cubs can be observed in smaller quantities throughout the year. Laurenson et al. (1992) have also reported seasonal tendencies of free-ranging cheetahs in Serengeti with considerably more litters being born during the wet season. Possible seasonality of Tanzanian cheetahs had previously been suggested in another study revealing that none of 14 sample litters was produced between September and December (Schaller, 1972 in Laurenson et al., 1992). Caro (1994) suggested the seeming seasonality pattern of cheetahs reproduction might be associated with the abundance of young Thompson‘s gazelles in the wet season, which, according to FitzGibbon (1990), represent relatively easy-to-catch prey for cheetahs.

Caro (1994) pointed out a significant difference between reproductive success in the wild and in captivity, where most (95 %) of the radio-collared free-ranging

females reproduced, while only about 16 % of captive females had been reported to reproduce in the same time period (Marker, 1987 in Caro, 1994). Laurenson et al. (1992) reported the reproductive rate of wild Serengeti cheetahs to be relatively high as approximately 80 % of adults had successfully produced offspring during their lifetime. 15 % of the monitored cheetahs were reported to fail to reproduce, which is significantly lower than data obtained from captivity showing infertility in nearly 85 % of females (Marker and O'Brien, 1989). In contrast, Kelly (2001) points that only a small part of the studied females on the Serengeti Plains successfully raised offspring that survived and further reproduced. Moreover, only approximately 6 % of all cubs born survived till independence on the Serengeti Plains (average age at independence is around 17 months (Laurenson et al., 1992; Kelly et al, 1998). The fact that cub mortality is extremely high in the wild is supported by the later study of Sinclair and Arcese (1995), where up to 95.2 % of young cheetahs were reported to die before they reached independence. The main factors causing young cheetah losses are predation (mainly lions and spotted hyenas (*Crocuta crocuta*) (Laurenson, 1994), followed by human activities, abandonment by mothers and unpredictable environmental events (Caro, 1994).

In contrast, Laurenson (1995) believes that there is an adaptation evolved in cheetahs to the high juvenile mortality rate, as their cubs grow very fast in comparison with other large felids. Furthermore it was reported from the Serengeti Plains that females mated again very soon after loosing their previous litter. In adult and young females the average interval between cubs lost and the next successful conception was estimated to 18.7 and 67.4 days, respectively, with the shortest pause being only 2 days (Laurenson et al., 1992). Caro (1994) reported resumption of estrus and occurence of mating within 3 weeks on average after the previous litter was lost. Although, according to Caro, normally cheetahs deliver their next litter after the previous offspring separates from the family, the majority of females were reported to conceive again while still accompained by their previous family (Kelly et al., 1998); or, in case of a tame free-living female, even give birth to the next litter before separation of previous offsprings (Adamson, 1969 in Caro, 1994).

Size of the known wild population is estimated to be around 7 000 individuals, while the total population hardly reaches the number of 10 000 adult individuals (Durant et al., 2008). Due to one or more bottlenecks through which cheetah population underwent in the past (Menotti-Raymond and O'Brien, 1993), genetic variability of this species is considerably low in comparison with other felids (O'Brien et al., 1986). Fragmentation of cheetah‘s habitat and so also its population into smaller isolated parts caused high level of inbreeding, resulting in increased homozygosity within each population (Merola, 1994).

2.1.4. Reproduction of cheetahs in captivity

The survival of many critically endangered mammalian species is often dependent on successful breeding in captivity that provides the future opportunity of reintroduction to the wild (Dehnhard et al., 2008). In contrast to the relatively high reproduction rate of wild cheetahs, captive breeding of the species has not experienced much success. Although cheetahs have been held in captivity since the pharaohs (Harper, 1946 in Caro, 1994), the first captive birth of cheetah cubs was reported in 1956 (Sunquist, 1992 in Caro, 1994). The poor reproductive success is demonstrated by statistics from North American facilities showing 17 of 108 females (15.7 %) and 12 of 85 males (14.1 %) had ever bred between 1871 and 1986 (Marker and O'Brien, 1989). Eventhough the only *in situ* breeding facilities are located in South Africa (Marker, 2002), in 1996, southern African cheetahs represented about one third of the world‘s captive population. Between 1970 and 1996, 772 cubs were born there. At the end of this period, the African captive population counted 262 animals in total – only 30 % of these came from wild, while 70 % were captive-born (Marker, 1998 in Marker, 2002). Eventhough some facilities have registered success in reproducing cheetahs, according to Dr. L. Marker (Cheetah International Studbook), who keeps records on all cheetahs in zoos and private facilities, the captive population is still not self-sustaining, and still requires importing new animals from the wild populations to be maintained. Unfortunately these wild populations are not considered as viable either from the long-term survival point of view. In order to maintain the wild population, successful and continuous breeding in captivity is essential (Marker, 1998).

Although female cheetahs are capable of reproducing at a very early age (the earliest around 13 to 16 months of age (Wrogemann, 1975)), in captivity a male is usually introduced to a female for the first time around 3 years of age (Bertschinger et al., 2008), which is the average age of first sexual activity in wild cheetah females reported by most authors (e.g. Caro, 1994; Kelly et al., 1998; Sunquist and Sunquist, 2002). In captivity, cheetahs are reported to live for 14 to 15 years (Caro, 1993), nevertheless, in captive-bred cheetahs the reproductive success is very low, and it even decreases in females 9 years of age and older (Crosier et al., 2009).

Because of the rarity of many felid species, captive breeding programmes often employ modern techniques of assisted reproduction to attempt to ensure maximal success (Wildt et al., 1981; Dehnhard et al., 2008). These techniques include hormonal induction of follicular development and ovulation, semen collection and artificial insemmination (Platz and Seager, 1977; Wildt et al., 1981). One of these techniques is hormonal induction of follicular development and ovulation. According to Wildt and colleagues (1981), the hormonal induction of ovarian activity for an artificial breeding programme can be achieved with a series of injections with follicle stimulating hormone (FSH) and human chorionic gonadotropin (hCG). In the study the attempted follicle growth and maturation failed in approximately 33 % of individuals (two of six females did not, for uncertain reason, demonstrate follicular activity). However, the gonadotropin treatment was successful in four cheetahs and one female even produced six mature, rupture capable follicles. The authors suggest that in cheetahs hCG-induced ovulation is activated within a range of 24 – 36 hours after the first hormone injection, which is at the same time as it was observed in domestic cats treated the same way (e.g. Sojka et al., 1970; Platz et al., 1978). Nevertheless, none of the studied cheetah females showed any signs of behaviours related to estrus (Wildt et al., 1981). Also equine chorionic gonadotropin (eCG) can be used for induction of ovulation. However, both hCG and eCG cannot be used frequently, as they are known to trigger production of anti-gonadotropin antibodies in felids, preventing responsivness of the ovaries to repeated gonadotropin treatment (Swanson et al., 1995).

Other assisted reproduction techniques involve semen collection and artificial insemmination (Conservation Centres for Species Survival). However, Bertschinger et al. (2008) disagree with artificial insemination and *in vitro* fertilization having any positive effect neither on fertility, nor increase of the reproduction rate, nor reduction of the generation interval in cheethas. The only practical usage of these methods appears to be the ability to control the exchange of genetic material and the spread of diseases.

It would be a great achievement to solve the mystery of cheetah reproduction in captivity so more females became receptive and more litters were born, all in a natural way with no artificial interventions needed. Propper stimulation of the female prior to the actual introduction of the breeding pair might be a powerful tool for captive breeding. This way estrus might be induced, which can simplify the breeding procedure (Caro, 1993). As reported by several authors, ovulation occured in some cases when females had only been in visual and olfactory contact with a male, but not mated (Bertschinger et al., 1984; Asa et al., 1992; Wildt et al., 1993; Brown et al., 1996).

2.1.4.1. Problems affecting captive cheetah population

Many breeding facilities describe cheetahs as very complicated in the terms of breeding (K. Meeks 2016, personal correspondence). Difficult detection of estrus being one of the difficulty factors (Caro, 1994). According to some authors cheetahs have so called “silent estrus” (Wildt et al., 1981; Sorenson, 1995 in Wielebnowski and Brown, 1998), familiarly known as “silent heat”, mostly known in species of the Bovidae family (Zduńczyk et al., 2009; Hagmanet al., 2011; Rao et al., 2013). This state is described as an estrus without any obvious external signs (Swanson et al, 1972), meaning the ovarian folicle is maturing and rupturing as usually but it is not accompained with typical behavioural signs or visible physiological changes (Jiang and Ott, 2010). Wielebnowski and Brown (1998) disagree with cheetahs having silent estrus as their research revealed certain behavioural patterns occuring in higher frequencies during the corresponding changes of estradiol levels. These were rolling, rubbing, object sniffing, vocalizing, and urine spraying. In case females do not show estrus behaviour,

Cupps (1985 in Caro, 1994) believes it can be triggered by moving females either within the facility or between zoos. Although while determining estrus based on behavioural observation, one has to keep in mind the individual differences in expressing such behaviours (Wielebnowski and Brown, 1998). Moreover, those subtle changes in behavioural frequencies are difficult or even impossible to detect, as some females do not show any estrus behaviour even when a male is present (Smitsonian's National Zoological Park and Conservation Biology Institute). Furthemore behaviour monitoring is time-consuming especially if it is required to be done continuously. That is why some facilities use male cheetahs to help them with the estrus detection in females. The male is allowed close to the female, usually behind a fence at first for safety reasons. If the male starts exhibiting sexual behaviour (e.g. stutter bark), the female is most likely in heat (Bloom Leeks, Smithsonian National Zoological Park). This technique is used in White Oak Conservation Center with great success (Meeks 2016, personal correspondence). In order to determine the right time for breeding, vaginal cytology or hormonal level measurements are sometimes implemented in breeding programmes (Bircher and Noble, 1997). It was revealed that cheetahs exhibit long periods of anestrus, during which the animals can appear to be acyclic, thus infertile. These periods of sexual inactivity usually last 2 to 5 months, therefore long-term monitoring of ovarian activity is necessary to distinguish between anestrus period and long-lasting acyclicity in females (Brown et al., 1996).

Another difficulty factor is that cheetah females are very selective about their mating partner (e.g. Bircher and Noble, 1997; Carmignani, 2014; Smitsonian's National Zoological Park and Conservation Biology Institute; Omaha's Henry Doorly Zoo & Aquarium). However, Karen Meeks (2016, personal correspondence), cheetah breeder from White Oak conservation and research facility in Florida, does not agree completely. Although they have experienced several cases when copulation did not happen, according to Meeks cheetah breeding is mostly an issue of propper timing rather than females‘ refined taste. “Most females, if ready to be bred, do not really care who breeds them.” Rejection of a male by a female is very rare in White Oak and is almost exclusively connected with breeding young “first time” females. Meeks has noticed a trend though that females preffer a male they have already mated with

before. The same observation was made in Saint Louis Zoo, where all breeding animals exhibit a strong mate preference. One female even bonded to a certain male and all attempts to breed her with other males failed each time (Bircher and Noble, 1997). According to many scientists, providing animals with the opportunity to choose their mating partner is an important part of successful breeding (e.g. Møller and Legendre, 2001; Gowaty et al., 2007; Clutton-Brock and McAuliffe, 2009). However zoos and other breeding facilities try to ensure genetic diversity and prevent inbreeding by composing mating pairs of unrelated individuals (Grisham, 1997), however sometimes a breeding manager's selection of a mating partner and a female's own choice may not be alligned. Such a conflict may then result in aggression rather than mating (Roberts and Gosling, 2004), or, in less serious cases, disinterest on female's part (Caro, 1994).

Another factor limiting captive population growth is the condition of the animals. O'Brien and others (1983; 1985; 1986) believe that the poor health of the captive (and wild) population is a consequence of population homogenity, caused by the low number of reproduction-capable individuals. As part of captive cheetahs is taken from the nature where cheetah population is devided into many small groups inhabiting isolated areas, the genetic variability within both wild and captive populations is very poor (Marker, 2002). High genetic monomorphism, which is typical for most endangered species, is demonstrated for instance by the lack of variety in the major histocompatibility complex (MHC) genes that encode immune responsiveness to viral infections and other pathogens. That is believed to cause such a great sensitivity of cheetahs to diseases caused by feline coronaviruses, which are barely lethal for domestic cats (Horzinek and Osterhaus, 1979), but highly dangerours for cheetahs. These viruses are believed to be the cause of high adult and juvenile mortality in captive population (O'Brien et al., 1983). Walzer and others (1995; 2003) report captive cheetahs suffer from unexplained ataxia[1] and paralysis, that might be associated with their genetic condition. O'Brien and colleagues (1986) also suspect the considerably low genetic variability is the cause of high frequency of morphological

[1] Poor coordination and unsteadiness due to the brain's failure to regulate the body's posture and the strength and direction of limb movements. (Medical dictionary, www.medterms.com).

abnormalities of spermatozoa (up to 71 % (Wildt et al., 1983)) and thus low conception rate.

Merola (1994) suggested that common problems such as infertility, reduced litter size, or increased susceptibility to diseases are more likely to be impacts of captivite conditions rahter than genetic depression. Speculations about captivity itself being the main problem causing infertility in captive female cheetahs had already been hinted upon even before. Laurenson and colleagues (1992) noticed that in contrast to high fertility of free-ranging cheetahs concluded from high number of cubs being born in the wild, females' ability to concieve in captivity is extremely low. They observed that in wild cheetahs increased food supply for a short period seemed to have a positive effect on estrus occurrance (simmilar to the "flushing" technique used in livestock breeding (e.g. Bellows et al., 1963 in Cupps, 1987)) and suggested that captive cheetahs may possibly be recieving an increased amount of nutrition in general. They concluded that the lack of occasional enhanced food amount and/or obesity of the animals might play an important role in reproduction failure. The first complex study concerning poor reproductive performance in captive cheetahs was conducted on 60 males and 68 females maintained in 18 different institutions throughout North America. In it, only 23 % of captive females turned out to have developped mature follicles (size 4 mm or bigger). The conclusion was that female infertility was most likely caused by inappropriate management and husbandry conditions (Wildt et al., 1993). This is supported by the finding that prevalence of some deadly disorders is significantly higher in captive-bred cheetahs than in wild population (e.g. prevalence of chronic gastritis is 64 % and 3 %, respectively (Munson, 1993)). In accordance, others add that the susceptibility of captive but not wild cheetahs to unusual diseases may be an outcome of chronic stress (possibly result of captivity), that modulates an immune response to pathogens (Munson et al., 2004) (Terio et al., 2004). Infant mortality in the wild is very high mainly due to high level of predation. In captivity, where this factor is elimintеd, around 20 – 40 % of cubs still die – most of them within the first month of life (Marker and O'Brien, 1989; Bertschinger et al., 2008). Although many deaths of cubs born in American facilities were unclear, some of the known causes of death were infection, stillbirth, congenital defect, canibalism and

maternal neglect (Marker-Kraus and Grisham, 1993). Arck and colleagues (1995) believe that stress might play an important role in maternal neglect, as it has been proven to negatively influence both the mother and her cubs.

2.1.4.2. Possible solutions to breeding problems

Management seems to be one of the most important factors reflecting in cheetah females' reproductive performance. Therefore a proper management of the animals appears to be the most effective tool in cheetah breeding. Practices proved to be helpful in successful reproduction in captivity are based on keeping animals in an environment as naturalistic as possible (Cupps, 1985 in Caro, 1994; Bircher and Noble, 1997). This includes for instance providing cheetahs with large enclosures and keeping potential prey species within detectable distance (Seager and Demorest, 1978; (Bircher and Noble, 1997). At the same time it is desirable to keep females out of sight of other large carnivores (Bircher and Noble, 1997; N. Hullet, pers. comm. in Caro, 1994). As females live solitary in nature, they should be housed in the same manner. According to Brown and colleagues (1996), females housed within physical proximity of other cheetahs appeared to negatively influence each other in the terms of breeding. They described a situation where females took turns in periods of ovarian activity within the same facility, suggesting that reproductive suppression may play a role in the estrus cyclicity of females. These findings are in accordance with the same effect found in some other wild species such as African wild dog (*Lycaon pictus*, (Fuller et al., 1992), dwarf mongoose (*Helogale parvula*, (Creel et al., 1992) and Callitrichid primates (Abbott et al., 1998; Eppleand Katz, 1984; (French et al., 1984). Also continuous male presence may have a disfavourable effect on the reproductive performance of captive cheetahs resulting in a decreased libido in both sexes and reduced breeding success, as proposed in several studies (e.g. Brown et al., 1996; Wielebnowski and Brown, 1998; Meltzer, 1999). Therefore, if a male is introduced to a female's enclosure, his presence should only be short and only if at least one of the cheetahs shows sexual behaviour (Bircher and Noble, 1997). Caro (1993) proposed males should be housed together in pairs or triads to immitate nature conditions. Occasional inter-male aggression might also have a possitive effect on estrus occurence and female's willingness to mate (Bircher and Noble, 1997; Herdman 1972 in Caro, 1994). Keeping stress level at a

minimum is especially important for (a) pregnant females to prevent abortions (Arck et al., 1995) and (b) females with cubs to prevent maternal neglect (Laurenson, 1993) or killing the litter (Marker-Kraus and Grisham, 1993). The stress relief precautions include the reduction of noise and other disturbances and the provisioning of enough hiding places and dens (Laurenson, 1993)

Mate choice, which appears to be an important behavioural aspect of animal breeding (e.g. Møller and Legendre, 2001), is not always technically possible for capacity reasons. Therefore Roberts and Gosling (2004) proposed that the manipulation of females by altering "male's social context" may lead to an increased breeding success of artificially created pairs. By transferring scent of the preferred male onto a cage of a non-preferred male the researchers were able to influence female's initial preference. Although it still needs to be assesed whether this technique could be applicable for cheetah breeding.

2.2. Endocrine monitoring of ovarian activity in felids

Hormone monitoring proved to be a reliable and relatively cheap alternative way of monitoring the ovarian cycle in felids and other species. The estrus indicators that need to be measured are estrogens (mainly 17-β-estradiol), progesterone and their metabolites (Domènech et al., 2011). There is a generally significant variability in the ovarian steroid cyclicity within individual felid species and also whithin studied individuals, which can partially be explained by the fact that felids are induced ovulators. Therefore beginning and termination of estrous is regulated in slightly different way than it is in species ovulating spontaneously (Brown et al., 1996).

The lag time of steroid hormones from when they enter the bloodstream to the time they are excreted in faeces is around 48 h in felids. It can vary among individual animals due to several factors such as variation in diet, healt condition and metabolic rate (Brown et al., 1994; Wielebnowski and Brown, 1998).

Conventional methods used for monitoring of ovarian activity in domestic and wild species are based on the collection and analysis of blood samples. However, this method does not seem to be appropriate for wild felids, because they, same as most

of other wildlife species, are very sensitive to stress during invasive procedures (Brown et al., 1994). Especially during pregnancy, repeated blood sampling and/or ultrasound diagnosis, commonly used in domestic species, are not suitable for wildlife animals (Adachi et al., 2011). The stress caused during these procedures has a negative effect on both mother and foetus and is known to cause abortions in animals (Arck et al., 1995).

A convenient alternative for monitoring hormone levels is a variety of are non-invasive methods, usually based on saliva, urine or feces examination. The measuring of estradiol and progesteron metabolites has proven to be useful in the determination of estrus cycle, pregnancy and seasonal patterns of reproduction in some of ungulate, equid and primate species (Heistermann et al., 1993; Schwarzenberger et al., 1993; Bamberg et al., 1991; Lasley and Kirkpatrick, 1991; Hindle and Hodges, 1990; Reslir et al., 1987). In light of the fact that many felid species excrete urine by spraying, it is very difficult to collect it from pooled sources as it is normally done in other species (Brown et al., 1994). Shille et al. (1984; 1990) studied the steriod metabolism of felids by injecting readiolabeled steroids into a domestic cat and revealed the excretory pathway of estradiol. These studies have shown that more than 95 % of this hormone is excreted by feces. Similar studies suggested that estradiol and/or progesterone metabolites can also be measured in feces of non-domestic felid species such as tigers (*Panthera tigris*), lions, caracals (*Felis caracal*) (Graham et al., 1992), servals (*Felis serval*), bobcats (*Felis rufus*) (Shille et al., 1991), and cheetahs as well (Czekala et al., 1994) (Graham et al. 1992). Thus, non-invasive fecal steroid monitoring serves as a great tool for detecting and monitoring ovarian activity and pregnancy in stress suspectible species (Schwarzenberger, 2007).

2.2.1. Fecal steroid extraction in felids

As each taxa differs in morphology and physiology, so does the method for obtaining endocrine data depends on what species is studied. Brown et al. (1994) developed a non-invasive method for fecal steroid extraction especially suitable for felids. This method was based on a technique used for baboons (Wasser et al., 1991), only simplified and adjusted for felid species. The main changes were applied to the

boiling section, when ethanol used as boiling medium was diluted with distilled water to obtain only 90 % of the concentration instead of 100 % and at the same time the solution volume was lowered from 10 ml to 5 ml. By adding the water the extraction of estradiol increased by 20 %, while in progesterone metabolites the efficiency increased by 50 % (Brown et al., 1994). This effect was observed most likely because conjugated steroids, contained in feline species, are soluble in aqueous solutions (Touma and Palme, 2005). Brown et al. (1994) attempted to enhance the steroid extraction efficiency even more by increasing the percentage of water in the ethanol boiling phase up to 20, 30, 40, or 50 %, however, the results were not satisfactory as the only effect that was observed was a prolonged drying time. Another improvement included elimination of lipid removal ensured by two dichloromethan extractions from the procedure. Thanks to this modification the sample preparation time was markedly reduced, as well as the need for toxic chemicals.

Brown et al. (1994) point out that special attention is required during sample collection and preparation. They suggest that the fecal samples should be collected frequently, with three times a week as a minimum rate, because of the daily variation in concentrations of estradiol and progesterone metabolites in feces. Even samples collected daily exhibited variability in steroid metabolites not only within the studied group, but also within individuals (Brown et al., 1996). However, as other authors suggest, such variability in ovarian activity is common for cheetahs and other members of the Felidae family (Bonney et al., 1981; Seal et al., 1985; Schmidt et al., 1988; Yamada and Durrant, 1989; Asa et al., 1992; Schmidt et al., 1993; Brown et al., 1995; Graham et al., 1995). Especially in induced ovulators, such as cheetah or domestic cat, the steroid level variability may be related to this characteristics (Brown et al., 1996). Risler et al. (1987 in (Brown et al., 1994)) also suggested that the collected fecal samples should be mixed properly, especially if they are processed wet, because the steroid metabolites are not distributed evenly throughout the sample. This led the authors to create a recommendation suggesting the usage of well mixed dried fecal samples instead of wet faeces, as results obtained this way appeared to be more accurate. The greatest divergence in hormonal levels was detected in samples where large amounts of hair residue were found within (Brown et al., 1994). That is why it is

necessary to remove apparent extraneous particles such as hairs, grass, or stones before testing the samples.

2.2.2. Methods of non-invasive monitoring of sex steroids in felids

2.2.2.1. Immunoassay methods

Immunoassays are sophisticated bioanalytical methods for measuring the quantity of various substances such as hormones, proteins, antigens, viruses and others (Wild, 2013). Their development by Solomon Berson and Rosalyn Yalow is dated to late 1950s (Law, 2002). A technique sufficiently sensitive to determine concentration of steriod hormones – namely 17-β-etsradiol – was first successfully developped in 1969 (Abraham, 1969). A short time after immunoassay development and usage dramatically expanded, with this method being employed in general analytical laboratories (Law, 2002).

Immunoassays have been used for wide variety of investigative work concerning dissease diagnosis, drug development, monitoring, and research (Findlay et al., 2000), food safety (Draisci et al., 2001), and many more. Immunoassays are favourite means of measuring hormonal levels in humans and animals, because they, in comparison with other methods, are relatively cheap and do not require highly trained personnel (Stanczyk et al., 2007).

The basic principle of an immunoassay is based on binding reaction of at least one reagent antibody to a specific structure of a target analyte. The antibody is usually labeled with an element that emits measurable signal, e.g. radioactivity (Wild, 2013).

There are several types of immunoassays, each of them is characterized by differences in reaction principles and number of used antibodies. Below, the most common types of immunoassays are described.

Competitive Immunoassays

In competitive design of immunoassay, the antigen (analyte) in the sample competes with the labelled antigen for a limited amount of binding sites (Cox et al., 2012). The concentration of sample antigen can be measured as it is inversely

proportional to the known amount of labelled antigen (Goldys, 2009). Thus, the weaker the signal, the higher the concentration of the analyte. Here only one antibody is used that is specific for the analyte. Therefore this method is often used for analysis of small molecules, or in case there is no pair of antibodies matching to the analyte antigen (Cox et al., 2012).

Competitive immunoassay can be designed in two formats – depending on whether the solid phase is coated with antibody or antigen (analyte), they are known as antigen-capture competitive assay and antibody-capture competitive assay, respectively (Darwish, 2006).

Non-competitive immunoassays

Non-competitive immunoassays, sometimes called two-site or sandwich immunoassays (Darwish, 2006), are characterized by the usage of two antibodies which bind to two different sites of the antigen (Cox et al., 2012). The reagent antibody, that is fixed on the plate (solid phase), is the capture antibody. The other antibody, that attaches to anoher specific part of the target molecule and serves as a signaling element, is called a tracer (Wild, 2013). These signal antibodies are usually labeled with either a radioactive isotope, a fluorescent element or an enzyme (Darwish, 2006). Unbound tracers are washed away (separation) and the concentration of analyte is measured from the intensity of the signal as it is directly proportional to the amount of labelled antibody (Wild, 2013). The sandwich concept of immunoassay is mainly suitable for larger molecules with more than one recognition site (Darwish, 2006), but there are also techniques applicable for determination of small molecules (Wild, 2013).

Labels

A variety of substances are used as label elements in immunoassy techniques. The first immunoassays developped used radioactive isotopes such as radioactive iodine as a label (Yalow and Berson, 1960). Radioiodine (^{125}I and ^{131}I) has been used since, and together with radioactive hydrogen (^{3}H, tritium (Folkers, 2006)) belongs to the most often used radiolabels in general, thanks to which this type of immunoassays

are entitled radioimmunoassays (RIAs) (Goldsmith, 1975). According to Stanczyk and colleagues (2007), radioimmunoassays are the most often used method in steroid hormones studies. However, in steroid hormone analysis is, due to their low concetration in plasma, the selection of radioisotopes limited almost exclusively to radioiodine (Goldsmith, 1975).

In order to avoid the need for dangerous radioactive materials that requires special permissions and numerous safety precautions, radioisotopes were replaced with enzymes (Lequin, 2005). This kind of immunoassay was developped simultaneously by two different groups – as an Enzyme immunoassay (EIA) in Netherlands (Van Weemen and Schuurs, 1971), and as an Enzyme-linked immunosorbent assay (ELISA) in Sweeden (Engvall and Perlmann, 1971). Scientists were sceptical about enzymes succesfully replacing radioactive elements as labels, because of their significantly larger size (Lequin, 2005). However, according to Munro and Stabenfeldt (1984), EIA appears to be as reliable and precise in steroid analysis as RIA, with the advantage of reducing the time needed for the procedure. The principle of the enzymatic labelling is a reaction between enzyme and suitable substrate (chromogen) which gives off a special byproduct, that can be easily detected (Wild, 2013). There are several enzymes used in EIAs which, in combination with appropriate substrate, produce a coloured, luminescent, or fluorescent products (Cox et al., 2012). The most frequently used are horseradish peroxidase giving a red, brown or black colour, and alkaline phosphatase giving a blue or red colour (Wild, 2013).

Fluoroimmunoassays (FIAs) are simmilar to radioimmunoassays, only fluorophore is used as a label rather than a radioactive element (Darwish, 2006).

2.2.2.2. Mass spectrometry

More and more often mass spectrometry is used in larger laboratories, replacing traditional radioimmunoassays and direct immuno assays, becoming a standard tool for steroid hormone analysis (Stanczyk and Clarke, 2010). Some of the greatest advantages of the mass spectrometry method include: the ability of measuring multiple hormones within one analysis, a wider analytical range enabling analysis of highly complex chemical compounds, and an increased accuracy, which is

necessary for the determination of rare low-concentration elements (Field, 2013). Mass spectrometry in general is based on converting either organic or inorganic compounds into ionts, which are then saparated according to their mass-to-charge ratio, and both qualitatively and quantitatively determined. These actions happen under vacuum conditions in distinctive parts of the spectrometer: a ionization source, a mass analyser and a detector, respectively (Gross, 2006). The creation of the ions is achieved by electron ejection (an electron is ejected from a molecule, creating a positively charged ion), electron capture (an electrone is captured, resulting in a negatively charged ion), protonation (a proton is added to a molecule, creating a positively charged cation), cationization (a positively charged ion, such as Na^+, is added to a neutral molecule, creating a positively charged complex), deprotonation (a proton is removed from a molecule, creating a negatively charged anion), or by transferring a positively or negatively charged molecule from a condensed phase to the gas phase (possible only for precharged ions) (Scripps Center for Metabolomics and Mass Spectrometry). Numerous types of ionization methods have been developed and combined with various types of mass analysers. The mass spectrum is a plot representing mass-to-charge ratio (m/z) vs. intensity of a signal. The signal (or peak) intensity is a direct relfection of a relative abundance of the specific ion. The highest peak (most intensive signal) is called the base peak, which is usually considered 100 % relative intensity. The gap between two peaks represents the difference between the two m/z ratios (Gross, 2006).

Gas chromatography-mass spectrometry

Gas chromatography is a technique enabling the separation and the quantitative analysis of individual organic (and also several anorganic) components of the sample, that are in gaseous or liquid form. The gas chromatograph is composed of four main sections: the injection port, the column, the detector, the analyser and data acquisition system (electrometer and recorder). A very important part of the gas chromatograph is the carrier gas, which serves to push the sample gas forward. The carrier gas has to be inert, as not to react with the sample. The gasses usually used are helium, nitrogen, hydrogen or a mixture of methane and argon. The injection of the

sample can be executed manually or automaticly via an injection device. The column is the place where the separation of the components takes place as the sample goes through. A capilary column is the most often used type of column. It has a form of tube with the inner wall coated with either a solid porous material (gas-solid chromatography) or a viscous liquid material (gas-liquid chromatography). This coating is called the stationary phase. The following part is the detector. Its function is to distinguish the sample components from the carrier gas and transform that information into an elctrical signal, although "not all detectors respond to all components". The last part of a chromatograph is the data acquisition system which acts as a translator for the electrical signals to a peak chromatogram. The height of the peak is positively correlated with the signal intensity and thus the concentration of a certain component in a sample (Van Sant, 1997). Although gas chromatography is able to analyse a sample's components quantitatively, identification of individual components is not always precise. For this reason, gas chromatography is usually coupled with other analytical techniques such as mass spectrometry (Hites, 1997).

High performance liquid chromatography-mass spectrometry

High performance liquid chromatography is a useful tool for separation and analysis of small and thremo-labile molecules, that is why it is popular in steroid research (Brown and DeAntonis, 1997). Although because of a limited ability to identify some compounds, liquid chromatography is often combined with mass spectrometry. One of the greatest features of liquid chromatography-mass spectrometry (LC/MS) is the high sensitivity. Some steroids can be detected in concentrations lowel than <1 pg, which equals to 1 ng/dL from 0.1 mL of sample. Another time saving feature of LC/MS is the ability to simultaneously analyse several compounds widely ranging in concentration (McDonald et al., 2011). The sample injected into the chromatograph must be in liquid form, thus solid samples require dissolution in an appropriate solvent. A chromatograph consists of a solvent delivery system – a pump delivering a precise and constant pulseless flow of mobile phase from a reservoir. The injection device can be either manual or automated. The main part of the chromatograph is the column because this is where the separation takes place (Brown and DeAntonis, 1997).

The column is a tube usually made of stainless steel (McDonald et al., 2011) that is relatively resistant to corrosion. It is filled with stationary phase (Brown and DeAntonis, 1997). There are two basic principles of separation. The so called "normal phase", which combines polar stationary phase and low-polar mobile phase, and "reverse phase", which employs mobile phase (usually aquaeous solution) more polar than stationary phase. Due to the hydrophobic nature of all unconjugated steroids, they immediately attach to the hydrophobic stationary phase. The order in which the steroids are eluted can be roughly estimated from their structure – more polar steroids are released first, followed by less polar steroids (McDonald et al., 2011). These are monitored by a detector that creates an electric signal corresponding to the level of the analyte. The detector is connected to the data collection device (usually a computer), that represents the electrical signals in a form of chromatogram (Brown and DeAntonis, 1997).

Orbitrap

In this research a relatively modern mass analyser – Orbitrap – was used. The Orbitrap mass spectrometer is composed of an outer barel-shaped electrode and an inner spindle-shaped electrode which share a common axis and together create an electrostatic field. It's principle is based on trapping ionts orbiting around the inner electrode. The source of ionization in The Orbitrap used in this research is an electrospray ionizer (Hu et al., 2005). It serves to convert a sample's compounds into ionts without destroying them, which is an essential characteristics especially for processing thermo-labile chemicals (Fitzgerald and Siuzdak, 1996). The ions orbiting the central electrode also harmonically oscillate at the same time (Hu et al., 2005). The frequencies of the oscillations of the orbitally trapped ions are detected and transformed by a special function (Fourier transormation) into mass spectra (Makarov, 2000). The mass spectrum is then presented in a form of bar graph, histogram (profile spectrum), or, for higher accuracy, in a form of a table (Gross, 2006).

2.2.3. Hormonal profiles in felids

2.2.3.1. Estrus cycle

Length of the estrus cycle is calculated as a number of days between two estradiol peaks (Borque et al., 2005). Brown et al. (1996) considered the highest concentration of the elevated values to be the peak, assumed to be connected with estrus. Values generally ranging from 25 to 60 ng/g dry matter were considered as baseline concentrations, while peaks reached values from 100 to 750 ng/g dry matter. Estrogen level reaches the maximum concentration right before ovulation (Kwan, Tufts University). Progesterone remains at baseline concentration for the whole estrus cycle in absence of ovulation. The baseline levels of progesterone were reported to reach between 0.7 to 6 µg/g dry fecal matter (Brown et al., 1996). The maximum length of estrus cycle was determined to be 30 days – a period about twice longer than the average recorded cycle lenght (Eaton and Craig, 1973; Laurenson et al., 1992; Brown et al., 1996). The range of values exceeding this limit can be considered an acyclic period when ovaries become inactive. This state can last up to 5 months (Brown et al., 1996). Fig. 2 shows a hormonal profile during the estrus cycle of a female cat.

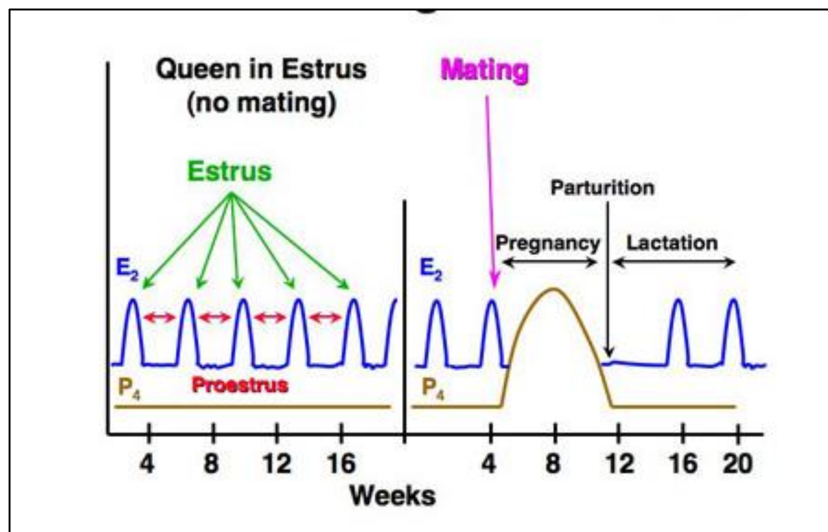

Figure 2 Hormonal changes in a female cat throughout the estrus cycle (University of Wisconsin-Madison, http://www.ansci.wisc.edu)

2.2.3.2. Pregnancy

As soon as about 24 hours after an LH surge and subsequent ovulation, significant elevations of progesterone concentration can be detected (England and von

Heimendahl, 2011). It continues to increase before reaching the peak concentration, which is usually around day 1 to 10 after ovulation (Brown et al., 1996). A postovulatory progesteron concentration increase is associated with increasing amount and size of cells composing CL (Hunter, 2003). During gestation, progesterone metabolite concentrations remain very high comparing to the baseline level. In pregnant females, the progestogen levels were measured to be 100 to 400 times higher than baseline values, which ranged between 0.7 and 6.0 µg/g. This state lasted until near parturiton and during that period progesterone concentrations almost never dropped beneath 20 times the baseline level. Females in which ovulation was induced hormonally by injecting eCG and pregnancy ensured by artificial insemmination, progesterone concentration remained generally high during the whole pregnancy span, however, there were considerable fluctulations throughout this period (Brown et al., 1996).

Estradiol concentrations remain low during pregnancy, except for one peak (approximately 10-fold elevation) occuring a few weeks before delivery. There is an apparent decline in estradiol concentrations once again after parturition (Brown et al., 1996).

2.2.3.3. Pseudo-pregnancy

Pseudo-pregnancy occurs when a female ovulates, but conception does not take place (England and von Heimendahl, 2011). An ovulated Graafian follicle is transformed into a functioning CL that ensures high level of circulating progesterone (Hunter, 2003). The hormonal profile in pseudo-pregnancy is very similar to that of pregnancy, however, the level of circulating progesterone starts to decrease again after reaching its peak instead of being maintained at a high level the whole time, as seen in pregnancy (England and von Heimendahl, 2011). The high production of progesterone thus lasts shorter during pseudo-pregnancy than during pregnancy – according to observations of Brown and colleagues (1996), the non-pregnant luteal phase ranges from 38 to 59 days, while the prengant luteal phase takes approximately twice longer – 94 days on average.

During a non-pregnant luteal phase generally low estradiol concentrations were interrupted by ocassional peaks, these occurences, howerver, appeared to be random (Brown et al., 1996).

Progesteron in its pure form occurs in faeces in very low concentrations or does not occur at all, therefore it is necessary to track progesterone metabolites for pregnancy monitoring. The levels of progestagens usually vary slightly throughout pregnancy, however, the composition is stable both within and among individuals (Adachi et al., 2011). If the foetus is aborted, progesteron levels immediately drop back to their base concetrations (Borque et al., 2005).

3. Aims of the thesis

This research was conducted in order to determine estradiol and progesterone metabolites excreted through faeces in cheetahs (*Acinonyx jubatus*).

The aim of the research was to preliminarly test whether it is possible to stimulate ovarian activity in cheetah females by conspecific male factors (scent, sound) using non-invasive methods of hormonal monitoring.

Additonal aim was to provide a brief summary of breeding situation of cheetahs in wild and in captivity and a compacted review of the use of non-invasive methods in sex steroid research concerning threatened felid species, with a special emphasis on cheetahs.

4. Material and methods

4.1. Animals

Three captive-born female cheetahs kept in the zoo Olomouc were involved in the study. All females were in reproductive age – older than 2.5 years (Bertschinger et al., 2008). Animals were housed in paddocks of various sizes (Abiba 360 m^2, Binty with two offsprings 1800 m^2, Gepina 250 m^2) in combination with inside enclosures. See Table 1 for detail information about the females. Abiba had visual contact through a mesh with the male from whom the fecal matter was taken. Binty and Gepina were inhabiting neighbouring paddocks without visual contact. Binty was accompained by her two approximately 7 month old cubs. All females were fed once a day. The diet consisted of chicken, rabbit, pork and beef meat and intestines. The detailed dietary plan is described in Table 2. All females had *ad libitum* access to water.

Table 1 Female cheetahs investigated in this study

Name	Birth Date	Nulliparous	Birth of Last Litter	Notes
Abiba	June 8, 2004	no	May 27, 2009	-
Binty	June 9, 2007	no	December 24, 2014	-
Gepina	May 27, 2009	yes	-	Hand reared

Table 2 Dietary plan of the studied cheetah females

Day	Abiba, Gepina	Binty[2]
Monday	Rabbit	Rabbit
Tuesday	Pork hearts	Chicken breast
Wednesday	Chicken breast	Chicken breast
Thursday	Beef meat	Chicken breast
Friday	Pork meat	Rabbit
Saturday	Chicken breast	Chicken breast
Sunday	Beef meat	Rabbit

[2] This female was allergic to beef and pork meat.

4.2. Fecal samples collection and stimulation of the females

Fecal samples were collected daily by a cheetah keeper 21 days before the experiment (control period), during the two-day experiment (from 1st to 2nd July 2015), and 21 days after experiment in a 44 day study period from June 10th to July 23rd 2015. The collection took place in the morning between 0900 and 1100 hr so the samples were never older than 24h (studied females usually defecate in the morning after waking up; Dostálová 2016, personal correspondence). The sampels were put into labelled plastic bags enabling future identification and frozen immediately after collection. They were stored at -20 °C until processing and analysis.

The two-day experiment was conducted in order to investigate whether the vocal and olfactory stimulation by a male can lead to significant changes in hormonal levels of females. The fresh fecal matter from a resident male was collected at the first day of the experiment and put into a plastic container. Several rather small punctures were made in the container so that the scent could go through the wall but direct contact of the animals and the content was prevented. The scent stimulus was placed into the female's enclosure while the female was inside her nightroom. After that she was let into her enclosure again and a stutter vocalization of an unknown male was played for 20 min for each female from 0800 to 0900, and later on again from 1400 to 1500. Behavioural responses were recorded during the whole time the vocalization was playing. After that, the scent container was removed from the enclosure. This was also repeated also on the following day.

4.3. Fecal steroid extraction

The extraction and hormonal analysis were carried out in the laboratory of the University of Chemistry and Technology in Prague under the supervision of trained personnel of the university and the Essence line company. Samples were thawed at room temperature and subsequently mechanically homogenized to ensure equal distribution of the steroidal components. A portion from each sample was withdrawn and the moisture content was determined using the Karl Fischer titration test. Using

the water content (%) and weight of the sample (g) the dry matter weight was calculated using the simple rule of proportion.

From each sample a portion of the fecal matter was withdrawn from three different sites of each sample to ensure a better spatial distribution of the steroids. The removed sample was weighed. 1 ml of ethanol (96 %) was added for each 0.1 g of dry matter weight according to the steroid solid extraction protocol, appended to the estradiol and progesterone DetectX immunoassay kit (Arbor Assays, USA). The test tubes with the mixture were put into an ultrasonic cleaner for 1 hour at room temperature (Sonorex digitec Bandelin, GmbH & Co. KG, Germany) to facilitate steroid transfer from the fecal material to the solution (ethanol). The samples were then centrifuged at 8,000 rpm for 15 minutes (Cooled centrifuge Universal 320R, Hettich, UK). The supernatant was removed and frozen (-80 °C). After processing all the samples, the supernatant solutions were thawed and stripped by nitrogen in order to evaporate ethanol. The samples were then redissolved in 400 µL methanol and subjected to further HPLC-MS/MS (high performance liquid chromatography-tandem mass spectrometry) analysis.

4.4. HPLC-MS/MS analysis

Methanolic samples were concentrated on the stationary phase of the column Bond elut C18. Prior to the sample introduction, the column was activated by rinsing 5 x 1 ml methanol and 3 x 1 ml aqueous solution of formic acid (0.05 m/L). Inserted samples (400 µL) were then lyophilized (evaporated to dryness under the liquid nitrogen) in a freeze-dryer (Trigon-plus RC) in order to concentrate non-volatile substances diluted in water.

100 µl LC-MS acetonitrile was added to the lyophilized samples and this mixture was shaken for 5 min (Minishaker IKA MS3 digital, GmbH & Co. KG, Germany) and subsequently centrifuged for 10 min (4 °C, 10,000 *g*) in order to retract substances that adhered on the test tube inner surface. Each sample was diluted in 50 µl solution composed of 70 % of acetonitrile and 30 % of water prior to the HPLC-MS/MS analysis.

4.4.1. Metabolic fingerprinting

Eighteen samples were selected analisis of which covered the whole time of the experiment. The days of stimulation (1.6. and 2.6.2015) were included in the sample set before stimulation due to the 48 h lag time of steroid hormone excretion into faeces (Brown et al., 1994). Selected days are listed in Table 3.

Chromatographic separation was done on the column Gemini C18 NX (bonded silica as a stationary phase). The mobile phase was composed of 10 mM aqueous solution of ammonium acetate (A) and LC-MS acetonitrile (B). The proportion of the mobile phases was as follows: 90 % of the phase A and 10 % of the phase B at the beginning of the analysis; after 5 min the proportion of the mobile phase A started decreasing linearly until it reached 10 %. During following 10 min the mobile phase A started raising linearly again until it reached 90 %. This concentration was then constant until the end of the analysis (30 min). The progression of the elution gradient is shown in Figure 2. The mobile phase flow was 150 µl/min and it was constant during the whole analysis. Samples were injected in the volume of 10 µl. The detection of individual substances was done using a mass spectrometer LTQ Orbitrap Velos, which was measuring within the range of *m/z* 80 – 1,500 Da.

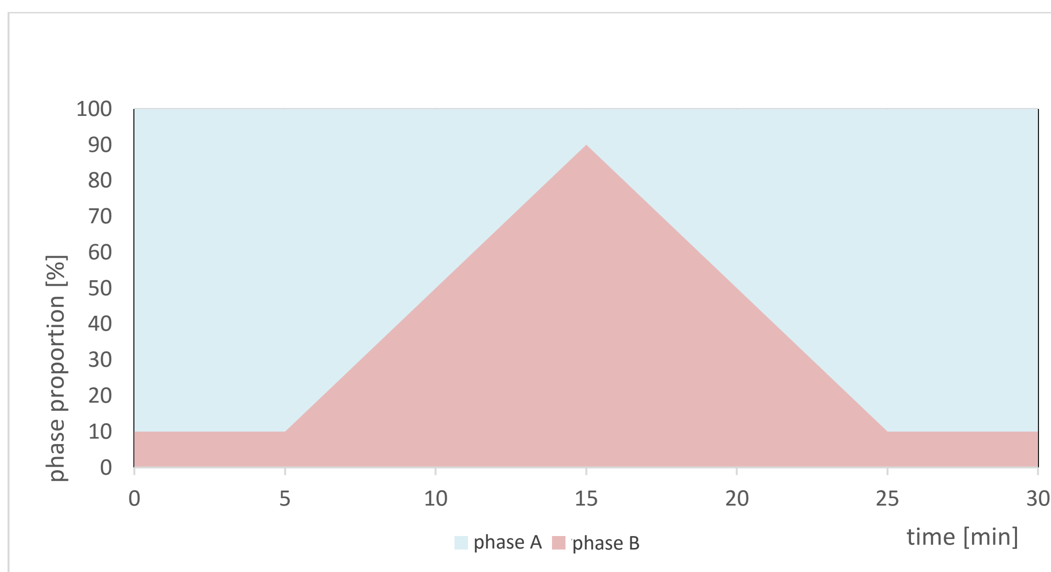

Figure 3 Elution gradient of LC method for steroid determination. Created by doc. Ing. Petr Kačer, Ph.D. (UCT Prague, 2016)

Metabolic fingerprinting was done in SIEVE 2.1. (Thermo Scientific, USA) to identify steroidal components present in the sample. Based on the obtained spectra, the identification of the most expressive peaks was done using Human Metabolome Database (www.hmdb.ca). The same database was used for selection of substances with connection to estradiol and progesterone metabolism from all identified substances.

Table 3 Dates of fecal samples selected for PCA analysis

Gepina (G1)	**Abiba (G2)**	**Binty (G3)**
10.6.2015	10.6.2015	11.6.2015
20.6.2015	20.6.2015	20.6.2015
2.7.2015	2.7.2015	2.7.2015
6.7.2015	8.7.2015	8.7.2015
14.7.2015	14.7.2015	12.7.2015
21.7.2015	21.7.2015	21.7.2015

4.4.2. Metabolic profiling

The separation of the substances was done by HPLC. Hypercarb column (porous graphitic carbon as a stationary phase) was used. The mobile phase was composed of 75 % of propan-2-ol with an addition of formic acid (1 % v/v) and 25 % of ammonium acetate. The composition of the mobile phase was constant during the analysis, as well as the mobile phase flow, which was 150 µl/min. Injected volume of the sample was 10 µl. Detection of the components was done by a tandem mass spectrometry (MS/MS) with an electrospray working in both positive ionization mode (ESI^+) and negative ionization mode (ESI^-). Analysis was done in Selected Reaction Monitoring (SRM) mode based on detection of concrete product ions.

Profiling (quantification) of steroidal substances was done in XLSTAT 2015 (Addinosoft, USA) sofware using Human Metabolomic Database. The obtained data were then processed in SAS Visual Analytics software and the outcomes were represented using dotted line graph.

4.5. Statistical analysis

The data obtained from metabolic fingerprinting were used for subsequent statistical analysis in SIEVE 2.1. software using Principle Component Analysis (PCA). This method was used for differentiating two sets of samples (before and after stimulation). The data were processed by Control Compare Trend (CCT) method with the emphasis on small molecules (molecular weight 70 – 1,500 Da). Prior to the analysis, the two data sets were discriminated by colour in the system, using red colour to represent samples before the stimulation and blue colour for samples after the stimulation. The output was a principal component scatter plot containing the information about the correlation of the samples before and after the stimulation.

The data from all collected samples obtained from the metabolic profiling were devided into five phases and analysed by Statistica CZ 12 (Stat Soft, Inc.). The phases are as follows: (1) before the stimulation (10.6. – 30.6.), (2) stimulation (1.7. – 3.7. due to the steroid excretion lag time), (3) 1 week after the stimulation (5.7. – 10.7.), (4) 2 weeks after the stimulation (11.7. – 17.7.) and (5) 3 weeks after stimulation (18.7. – 21.7.). The normal distribution was tested by the Shapiro-Wilk test and the Lilliefors test. Because the data did not show normal distrubution ($p < 0.01$), the differences among individual phases and females were tested by non-parametric statistics (Kruskal-Wallis test, Multiple comparison of p-values). The significance level was accepted at $p < 0.05$.

5. Results

5.1. Metabolic fingerprinting

Fifteen substances of steroidal character were identified in the samples by multicomponent screening. Their identity together with their chemical formula and *m/z* (mass to charge) ratio are presented in Table 4. Using Human Metabolome Database, only those steroid structures that are related to the metabolism of estradiol ad progesterone were selected for further analysis. They are presented in Table 5.

Table 4 Identified substances of steroidal character

<table>
<tr><th>Name</th><th>Chemical Formula</th><th>m/z</th></tr>
<tr><td>estradiol</td><td>$C_{16}H_{24}O_2$</td><td>272.1849</td></tr>
<tr><td>progesterone</td><td>$C_{21}H_{30}O_2$</td><td>314.2319</td></tr>
<tr><td>estradiol-3-sulphate</td><td>$C_{18}H_{24}O_5S$</td><td>352.1417</td></tr>
<tr><td>estrone</td><td>$C_{18}H_{22}O_2$</td><td>270.1614</td></tr>
<tr><td>estrone-3-sulphate</td><td>$C_{18}H_{22}O_5S$</td><td>350.1182</td></tr>
<tr><td>estriol</td><td>$C_{18}H_{24}O_3$</td><td>288.1798</td></tr>
<tr><td>20-OH-progesterone</td><td rowspan="4">$C_{21}H_{30}O_3$</td><td rowspan="4">330.2286</td></tr>
<tr><td>17-OH-progesterone</td></tr>
<tr><td>11-OH-progesterone</td></tr>
<tr><td>deoxycorticosterone</td></tr>
<tr><td>corticosterone</td><td>$C_{21}H_{30}O_4$</td><td>346.2217</td></tr>
<tr><td>pregnenolone</td><td>$C_{21}H_{32}O_2$</td><td>316.2475</td></tr>
<tr><td>5α-pregnanedione</td><td>$C_{21}H_{36}O_2$</td><td>320.2788</td></tr>
<tr><td>estrone-3-glucuronide</td><td>$C_{24}H_{30}O_8$</td><td>446.20132</td></tr>
<tr><td>estradiol-3-glucuronide</td><td>$C_{24}H_{32}O_8$</td><td>448.2170</td></tr>
</table>

Table 5 Selected steroid substances with a connection to estradiol and progesterone metabolism

<table>
<tr><th>Name</th><th>Chemical Formula</th><th>m/z</th></tr>
<tr><td>estradiol</td><td>$C_{16}H_{24}O_2$</td><td>272.1849</td></tr>
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<tr><td>estradiol-3-glucuronide</td><td>$C_{24}H_{32}O_8$</td><td>448.2170</td></tr>
</table>

5.2. Metabolic profiling

The average estradiol concentration of all samples (N = 115) was 187.78 ng/g dry matter, ranging from 84 to 444 ng/g dry matter. The average progesterone concentration of all samples (N = 115) was 34.38 ng/g dry matter, ranging from 5 to 74 ng/g dry matter. The measured concentrations means, ranges and standard errors (SE) are listed in Table 6.

Table 6 Fecal estradiol and progesterone concentrations in all studied cheetah females

Hormone	N	Mean (ng/g dry matter)	SE	Range (ng/g dry matter)
estradiol	115	187.7826	7.978	84 – 444
progesterone	115	34.3826	3.206	5 - 74

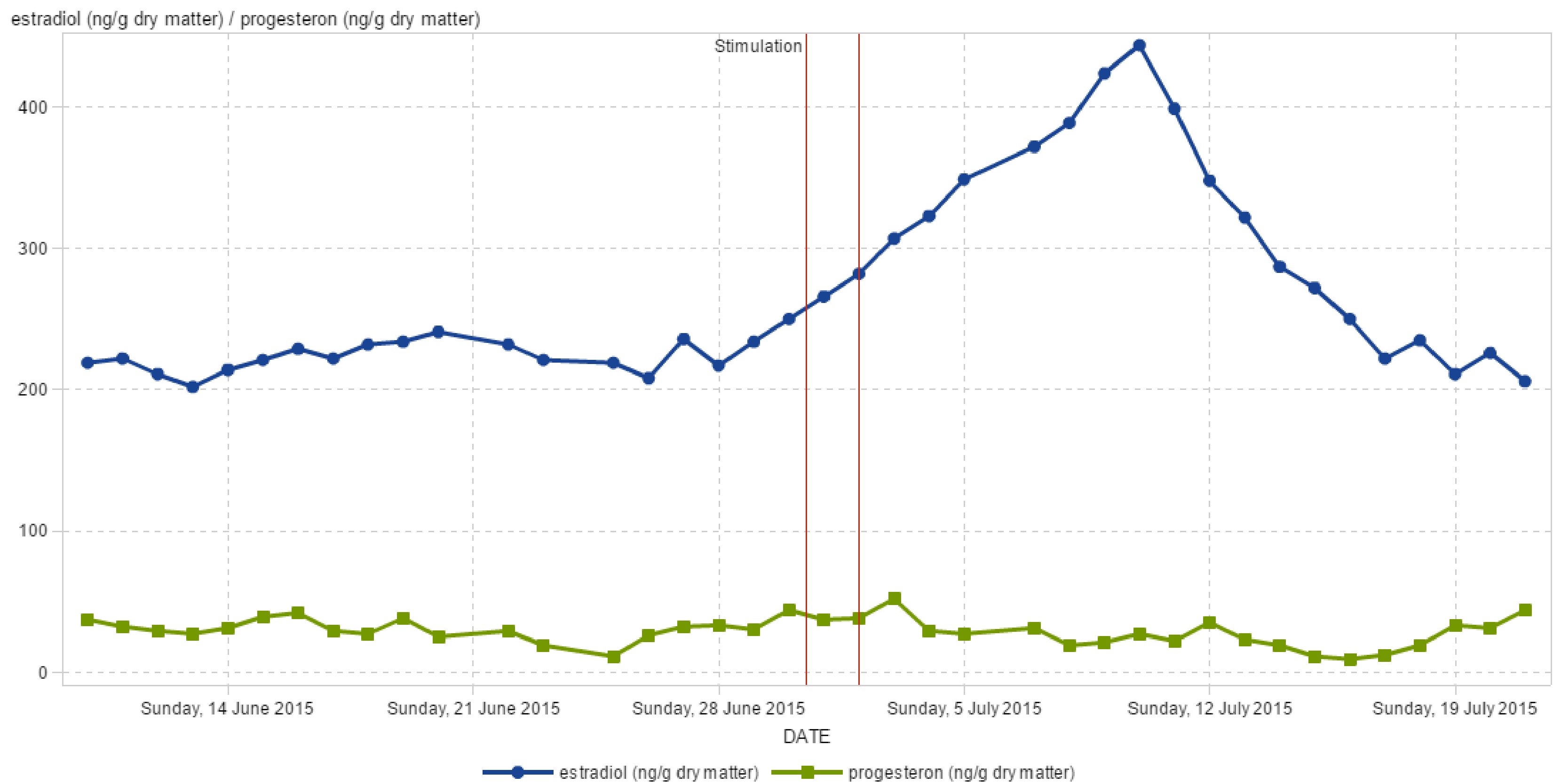

Figure 4 Estradiol and progesterone concentrations in fecal matter in Abiba

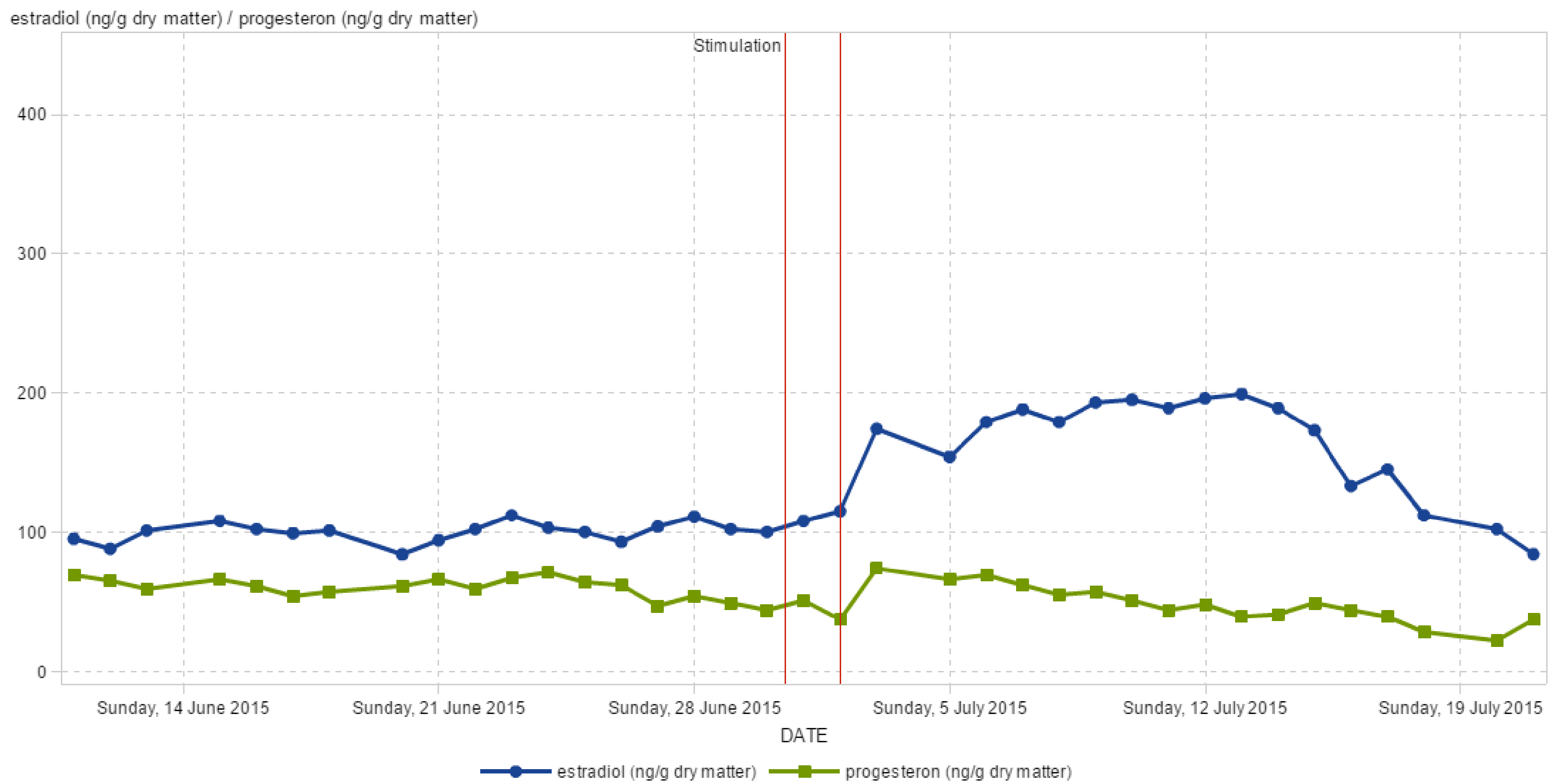

Figure 5 Estradiol and progesterone concentrations in fecal matter in Binty

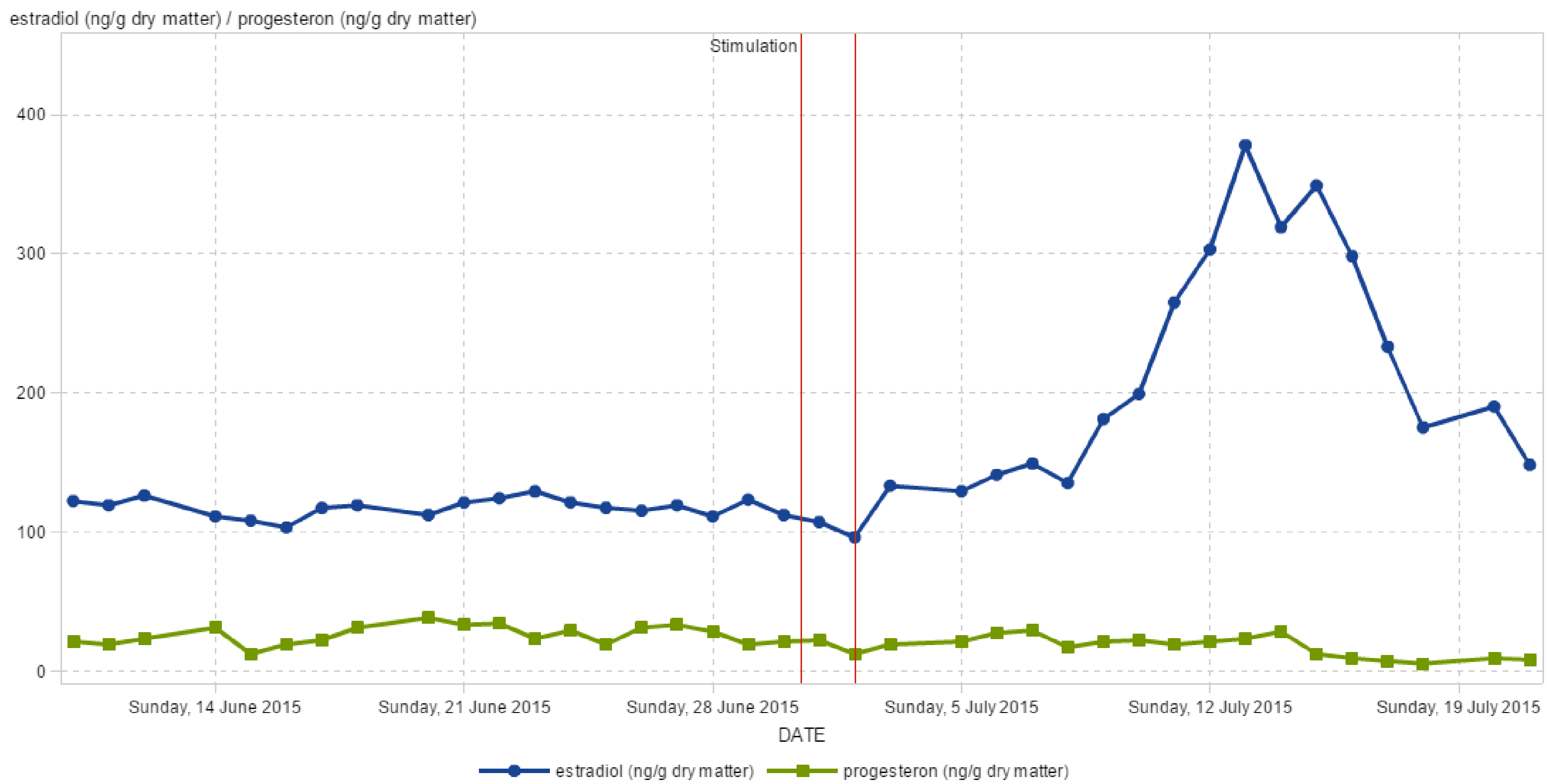

Figure 6 Estradiol and progesterone concentrations in fecal matter in Gepina

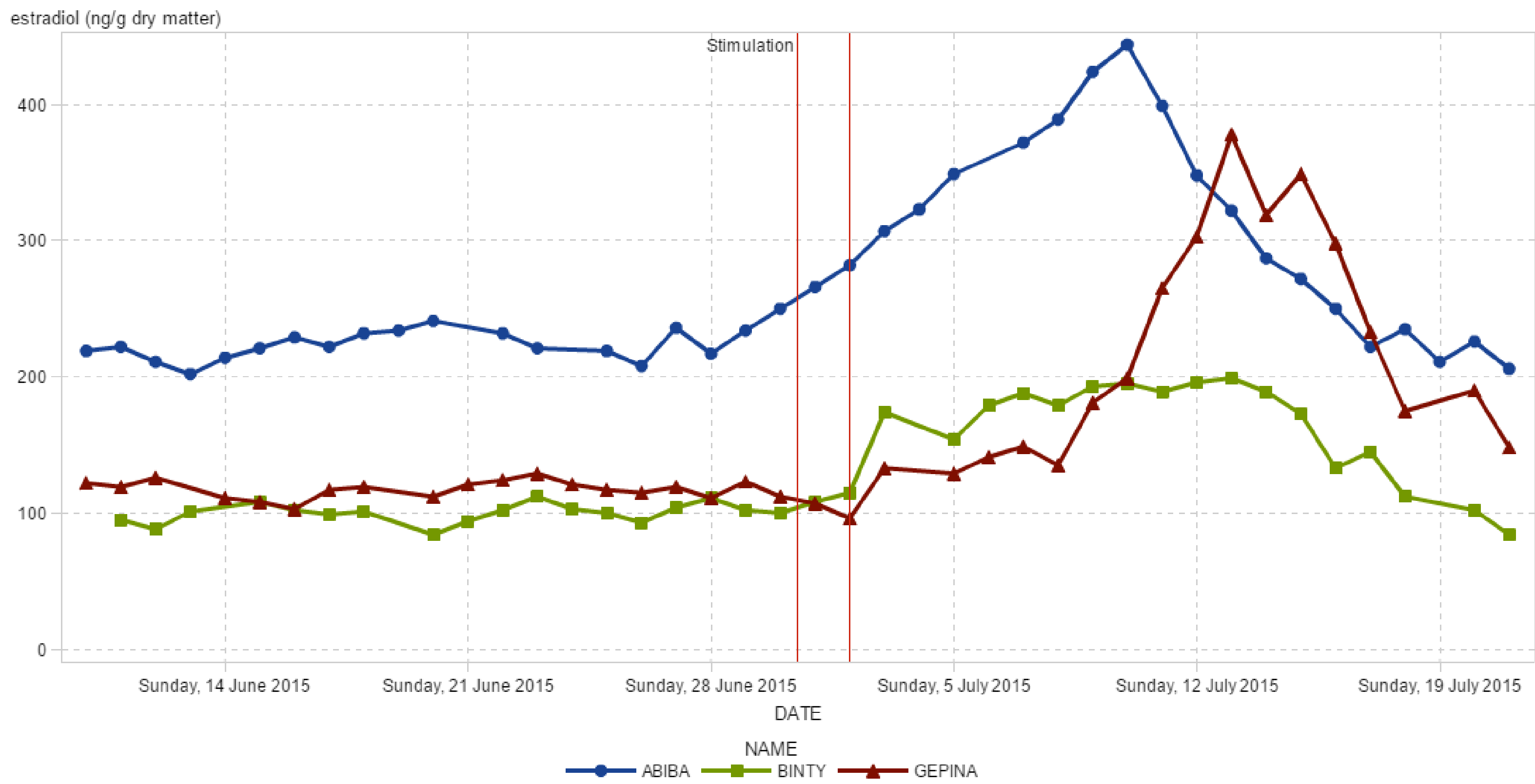

Figure 7 Comparison of the fecal estradiol concentrations in all three females

5.3. Statistical analysis

The results of the PCA (Figure 8) showed separation of the two clusters containig samples before (N = 18) and after (N = 18) stimulation. A partial separation can also be observed within the cluster marked as before stimulation (red colour), where the set of samples from Binty (G3) was separated from the sample sets of the other two females (G1 and G2).

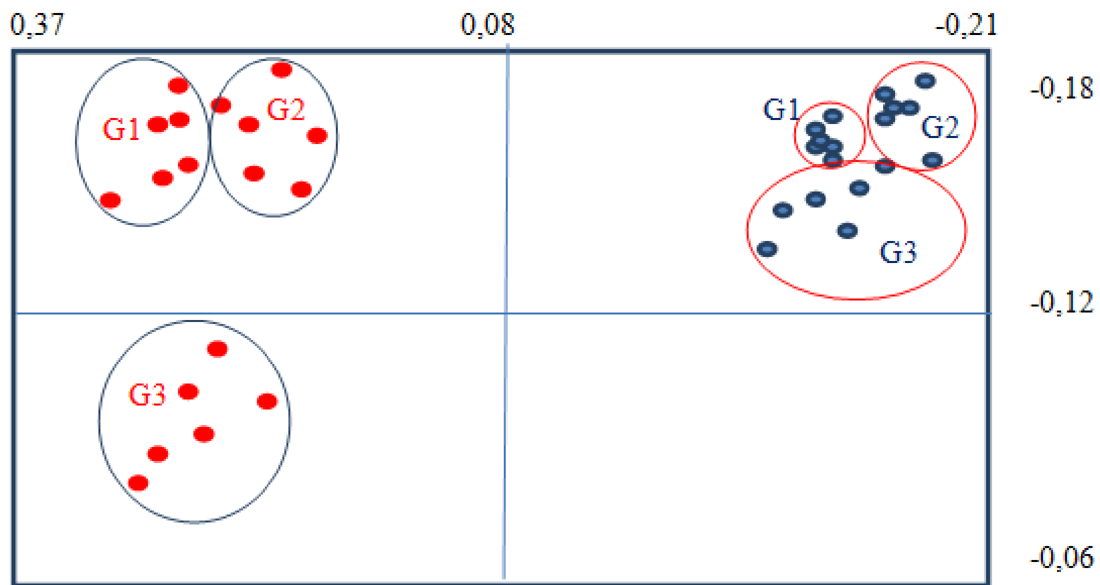

Figure 4 Separation of clusters before (red dots) and after (blue dots) stimulation by PCA analysis. Created by doc. Ing. Petr Kačer, Ph.D. (UCT Prague, 2016)

The Kruskal-Wallis ANOVA showed that the estradiol concentrations 1 and 2 weeks after the stimulation was significantly higher than the estradiol concentrations before the stimulation (Kruskal-Wallis test: H (4, N = 115) = 30.42660, $p < 0.0001$). See Figure 9.

Figures 10 – 12 show the comparison of estradiol concentrations in individual female cheetahs. The overall estradiol levels varied among the females (Kruskal-Wallis test, Multiple comparison of p-values: H (2, N = 115) = 64.1316, $p < 0.0001$ for Abiba vs. Binty and Abiba vs. Gepina, $p < 0.05$ for Binty vs. Gepina). As apparent from Figure 7, the estradiol concentrations raised after the stimulation and started declining again towards the end of the experiment in all three females. The overall estradiol level was

significantly higher in Abiba in comparison to the other two females (Kruskal-Wallis test, Multiple comparison of p-values: H (2, N = 115) = 64.1316, $p < 0.0001$).

The elevation of the estradiol level during first week after the stimulation was significantly higher in Abiba in comparison to Binty (Kruskal-Wallis test, Multiple comparison of p-values: H (2, N = 18) = 12.3285, $p < 0.05$) and Gepina ($p < 0.01$), but did not differ between Binty and Gepina ($p > 0.05$). See Figure 10.

The estradiol concentration during the second week after the stimulation was significantly lower in Binty than in the other two females (Kruskal-Wallis test, Multiple comparison of p-values: H (2, N = 18) = 13.4187, $p < 0.01$). The levels of estradiol did not significantly differ between Abiba and Gepina ($p > 0.05$). See Figure 11.

The estradiol concentration during the third week after the stimulation was higher in Abiba in comparison to Binty (Kruskal-Wallis test, Multiple comparison of p-values: H (2, N = 10) = 8.01818, $p < 0.05$), but did not differ between Abiba and Gepina ($p > 0.05$) nor between Binty and Gepina ($p > 0.05$). See Figure 12.

As apparent from Figure 13, the progesterone concentration did not significantly differ between any of the experimental phases (Kruskal-Wallis test: H (4, N = 115) = 10.29626, $p < 0.05$).

The progesterone concentrations during the first week after the stimulation were significantly higher in Binty in comparison to Abiba (Kruskal-Wallis test, Multiple comparison of p-values: H (2, N = 18) = 11.8321, $p < 0.05$) and Gepina ($p < 0.01$), but did not differ between Abiba and Gepina ($p > 0.05$). See Figure 14.

The progesterone concentration was significantly higher in Binty in comparison to Abiba and Gepina during the second week after the stimulation (Kruskal-Wallis test, Multiple comparison of p-values: H (2, N = 21) = 13.4625, $p < 0.01$), but did not differ between Abiba and Gepina ($p > 0.05$) See Figure 15.

The progesterone concentration did not differ significantly among females during the third week after the stimulation (Kruskal-Wallis test, Multiple comparison of p-values: H (2, N = 10) = 5.7909, $p > 0.05$). See Figure 16.

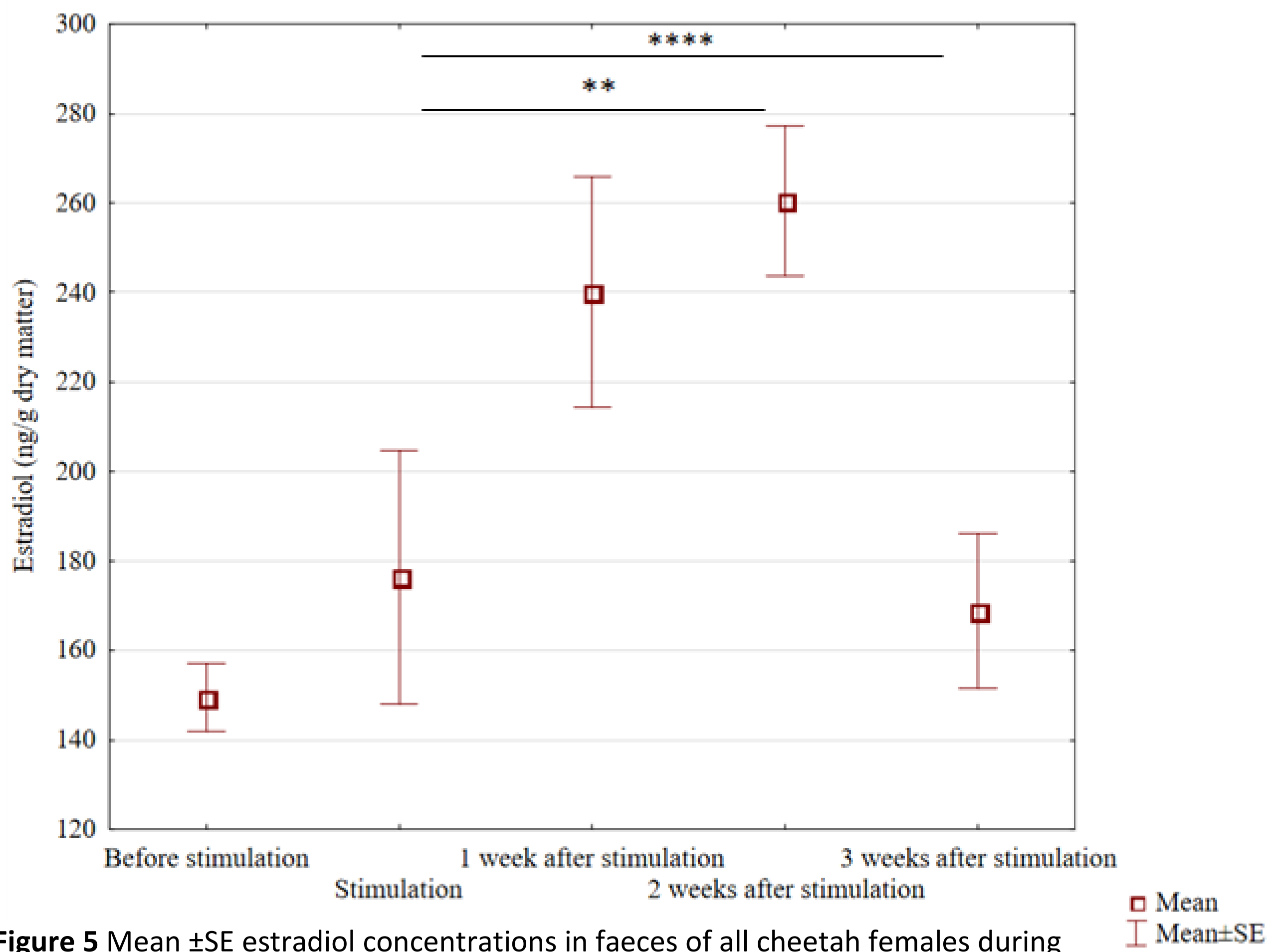

Figure 5 Mean ±SE estradiol concentrations in faeces of all cheetah females during individual experimental phases (** $P \leq 0.01$; **** $P \leq 0.0001$)

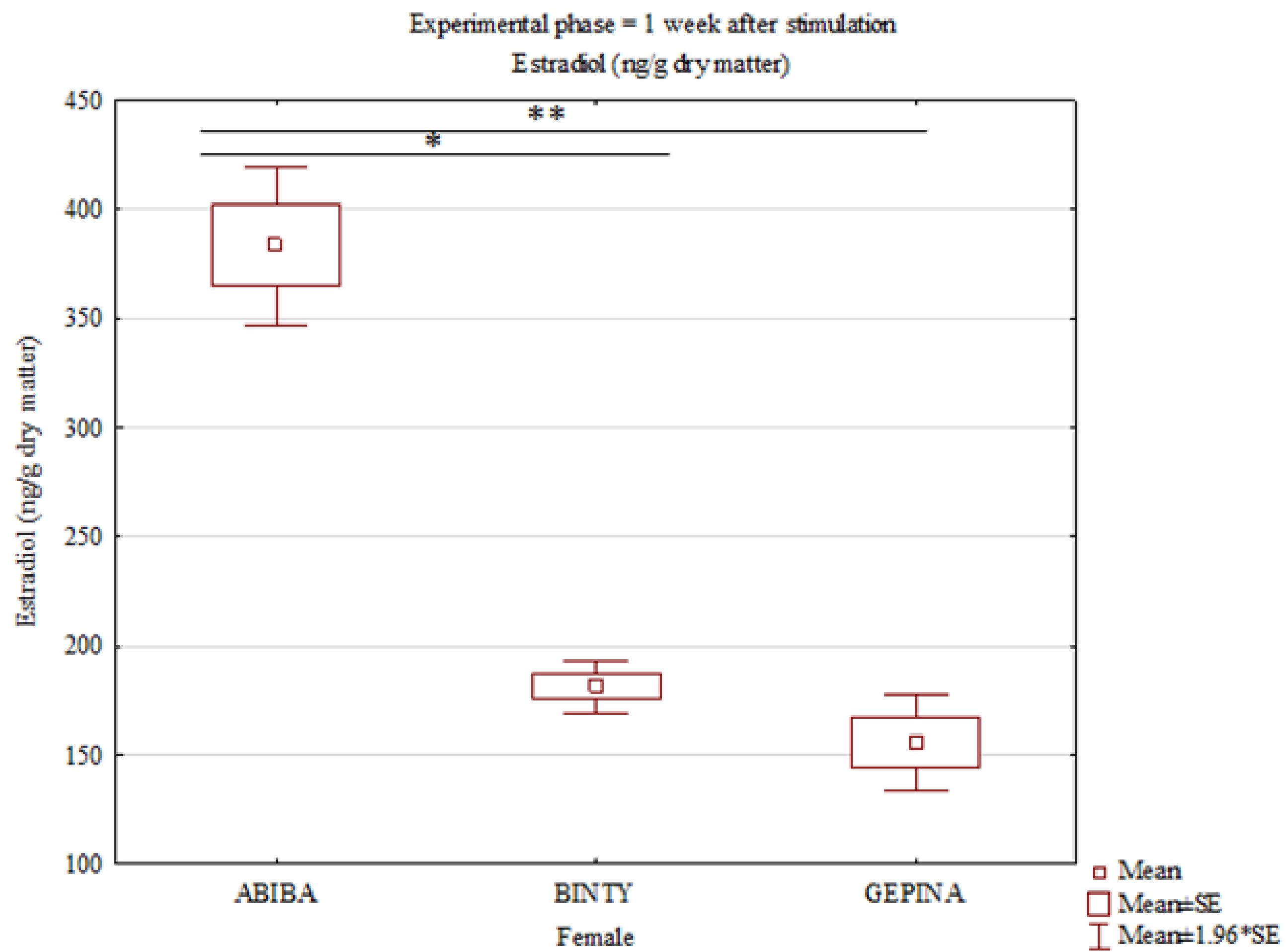

Figure 6 Differences among the fecal estradiol concentrations in the individual females during the first week after stimulation (* $P \leq 0.05$, ** $P \leq 0.01$)

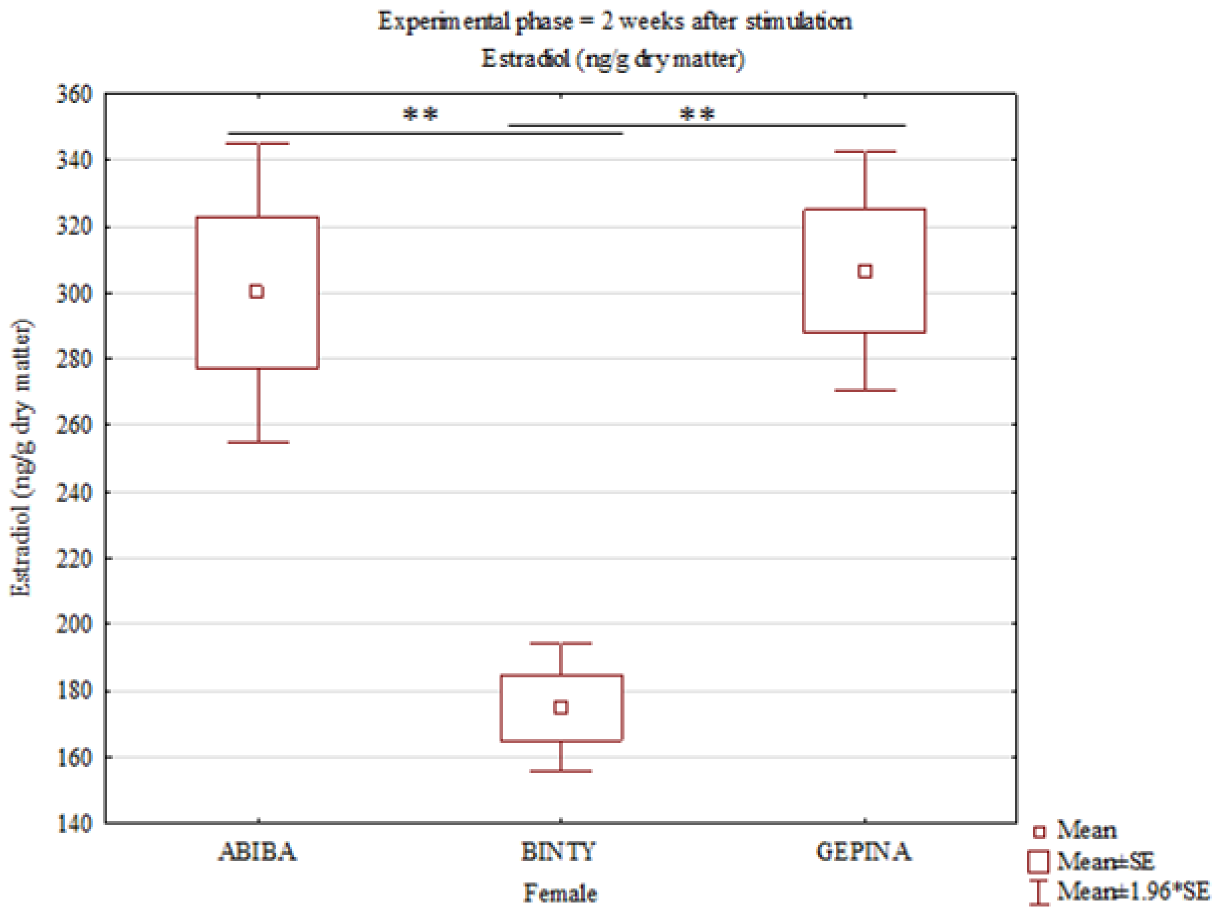

Figure 7 Differences among the fecal estradiol concentrations in the individual females during the second week after stimulation (** $P \leq 0.01$)

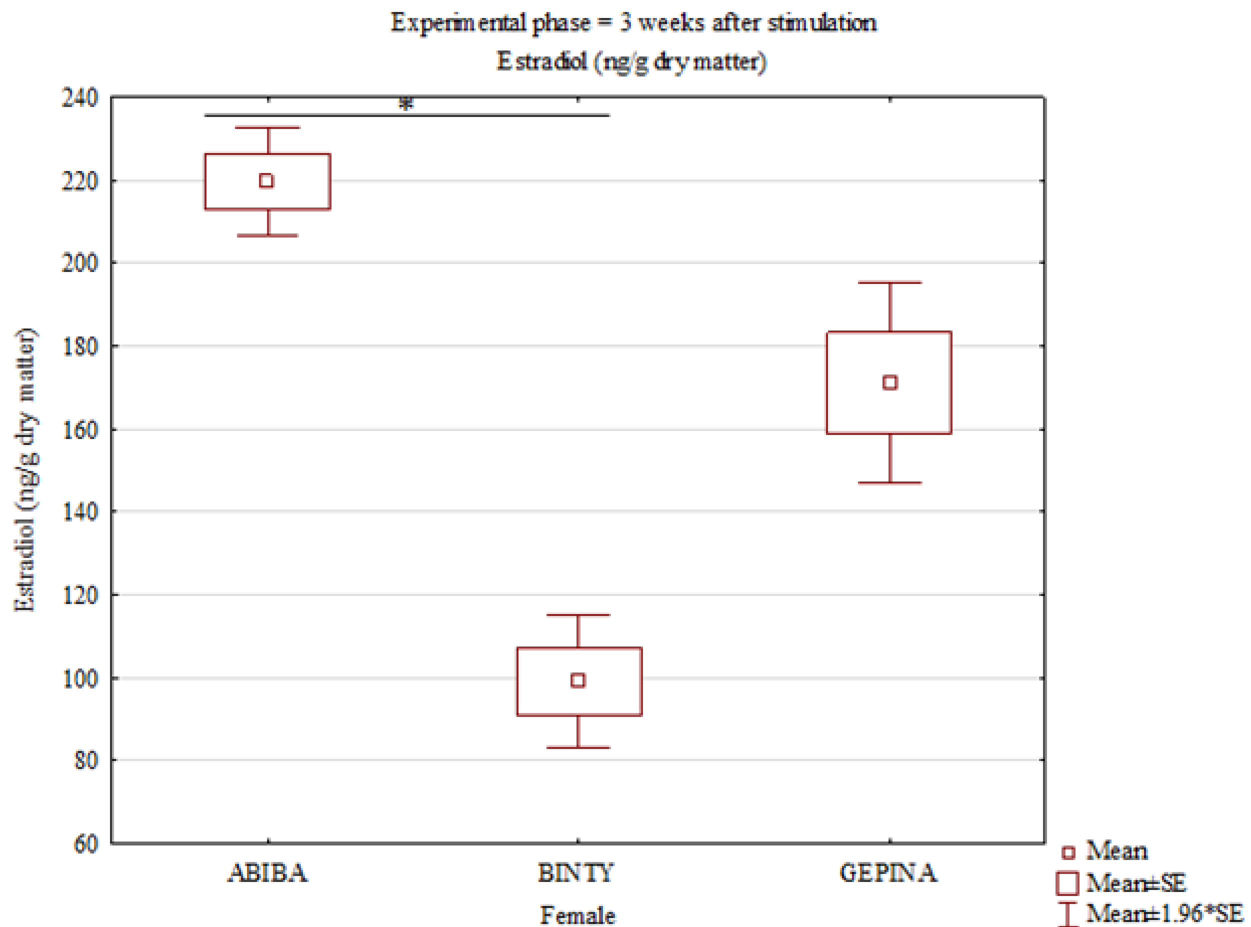

Figure 8 Differences among the fecal estradiol concentrations in the individual females during the third week after stimulation (* $P < 0.05$)

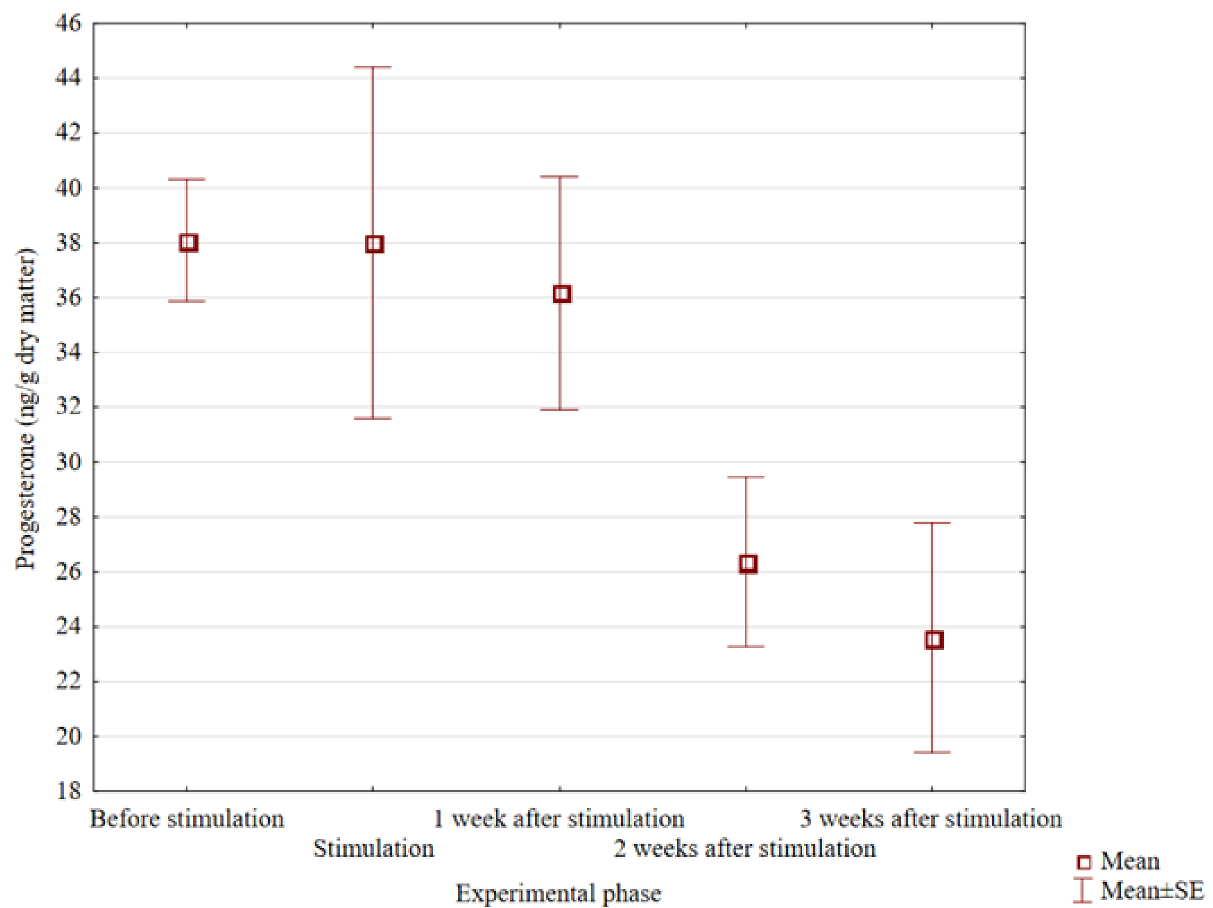

Figure 9 Mean ±SE progesterone concentrations in faeces of all cheetah females during individual experimental phases ($P > 0.05$)

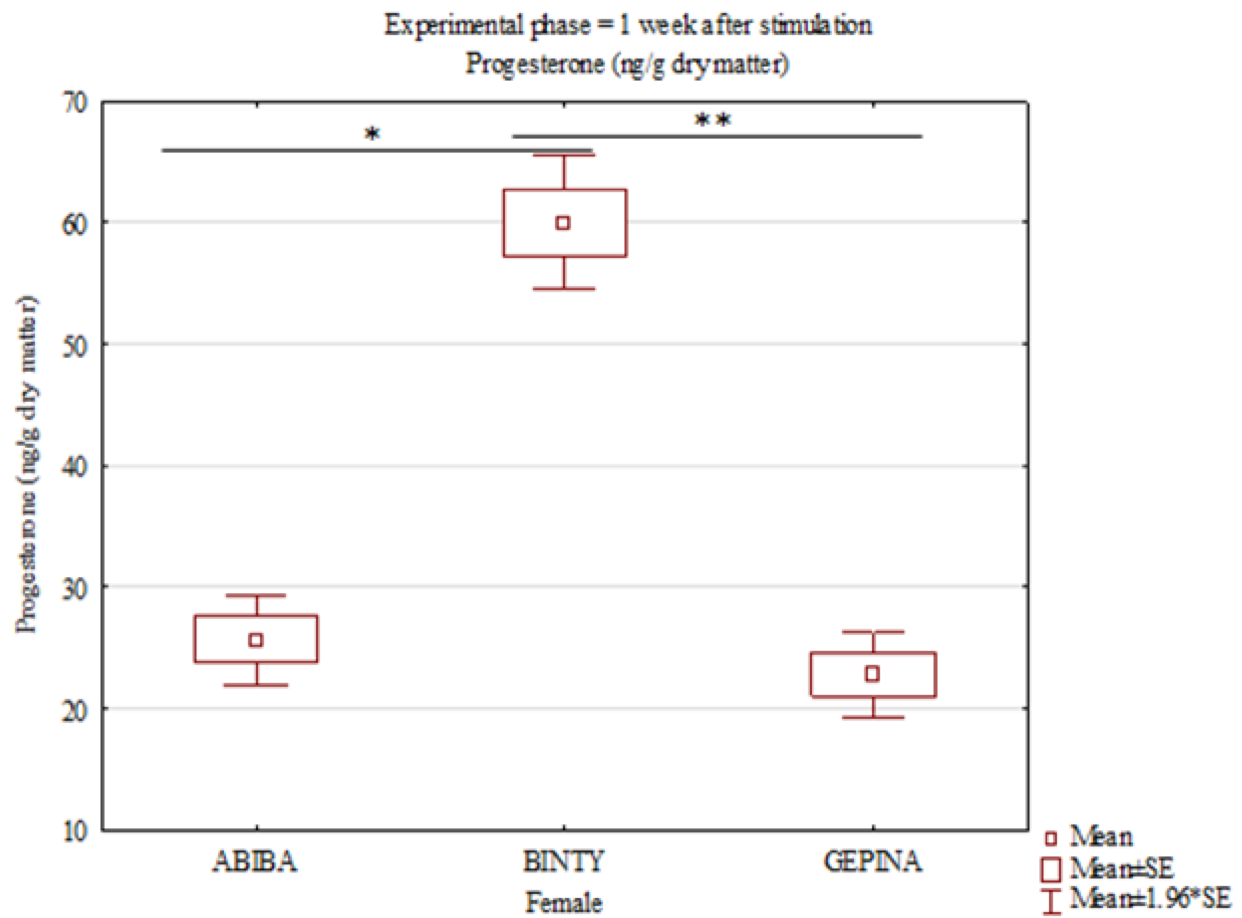

Figure 11 Differences among the fecal progesterone concentrations in the individual females during the first week after the stimulation (* $P < 0.05$, ** $P \leq 0.01$)

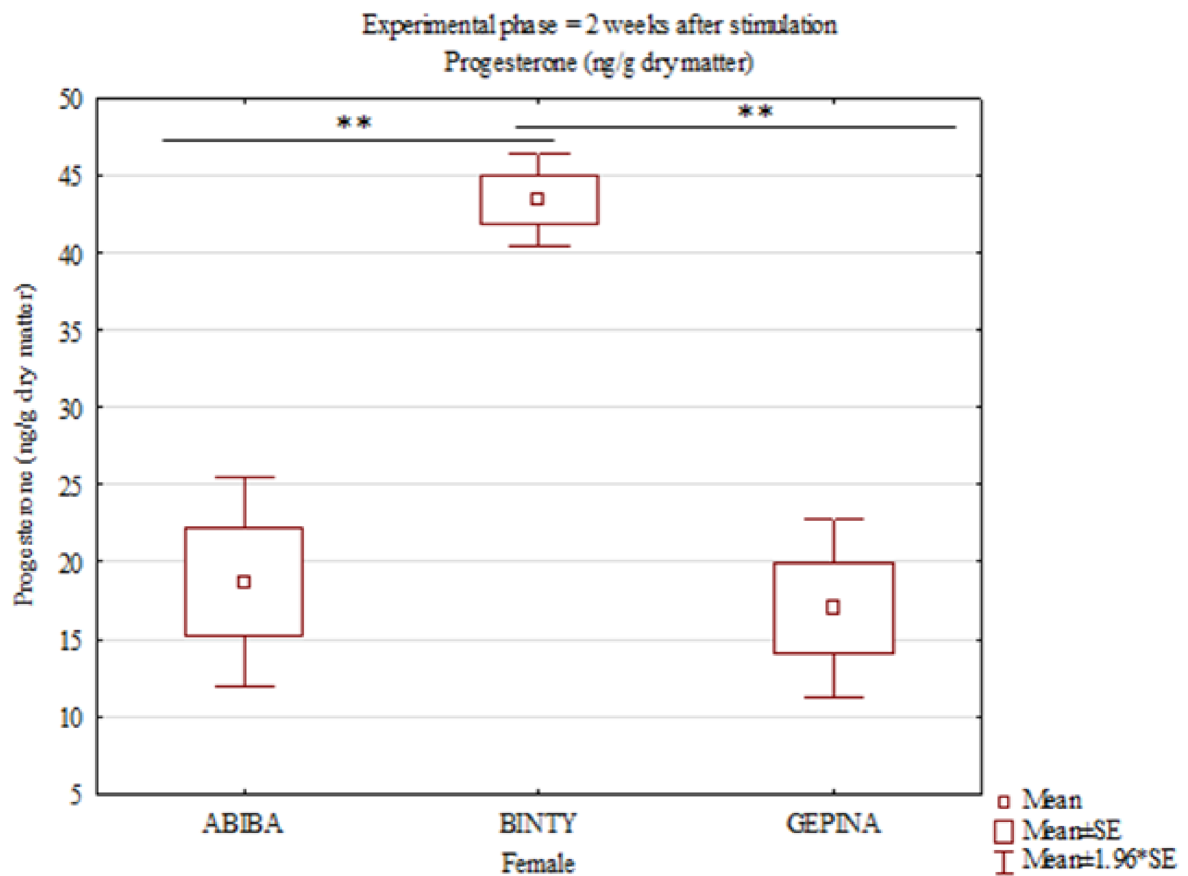

Figure 15 Differences among the fecal progesterone concentrations in the individual females during the second week after the stimulation (** $P \leq 0.01$)

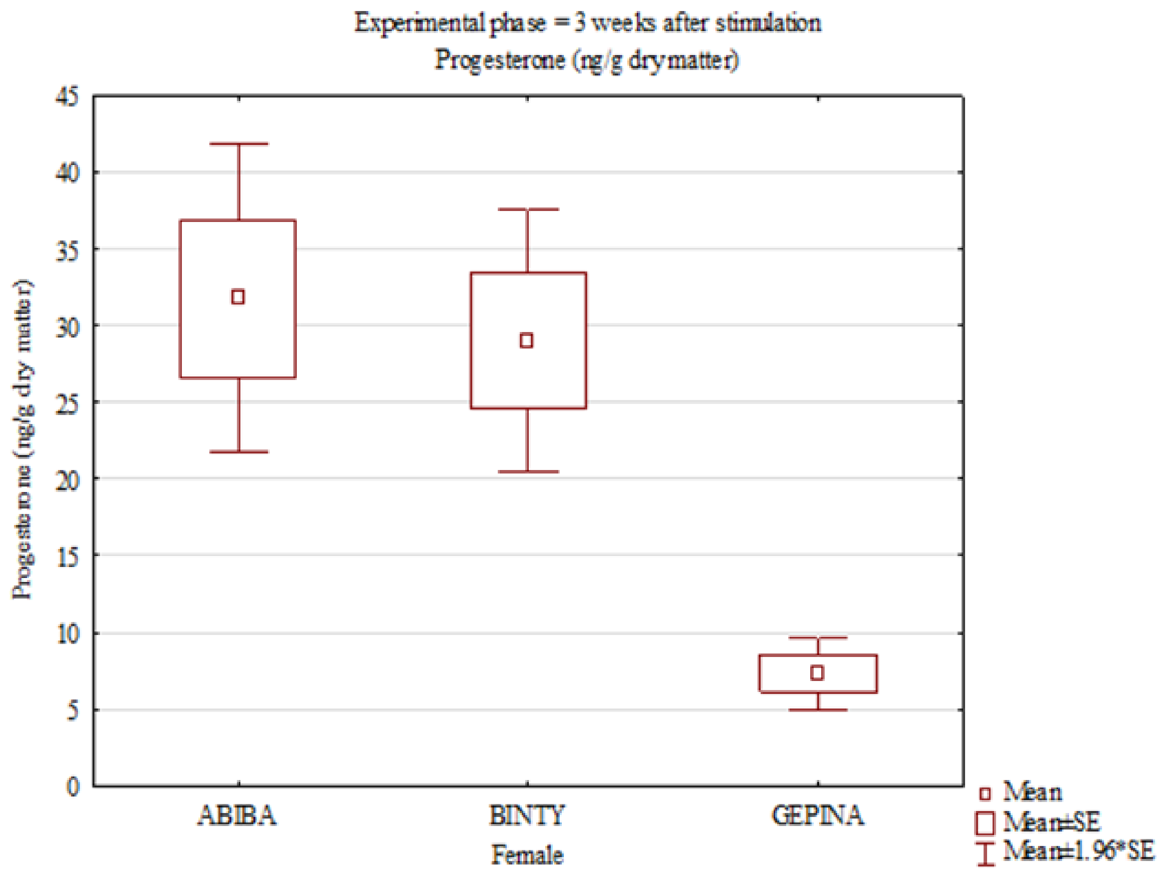

Figure 12 Differences among the fecal progesterone concentrations in the individual females during the third week after the stimulation ($P > 0.05$)

6. Discussion

This study is, to my best knowledge, the first attempt of a stimulation of a female cheetah by male factors (scent and voice). It has been suggested that stimulation of a female by a male might play an important role in the female's reproductive performance (Bircher and Noble, 1997). The separation of the two sets of samples (before and after stimulation) by PCA can be interpreted as an important metabolomic difference between samples before and after stimulation. The significant changes were detected in the estradiol level, which was elevated during two weeks after the stimulation. This suggests that a hormonal response was successfully triggered in the females by vocal and olfactory stimulation in in this study. It is, however, necessary to take into consideration the leght of the monitoring and the low number of individuals examined for this work; thus, it should be perceived as a preliminary study.

The partial separation of the group of samples from Binty (G3) within the set "before stimulation" could be assigned to the fact that this female was still accompained with her two cubs (7 months). It has been prevously reported from the wild that females still acompanied by their last litter are anyway able to concieve. Nevertheless, they do not give birth unless the previous offspring separates (Caro, 1994). If the average three month long gestation period (e.g. Brown et al., 1996) and the average 17 months of age of separating offspring (Kelly et al., 1998) are taken into account, then Binty had not been expected to show any prominent ovarian activity for another approximately 7 months.

The estradiol concentration measured by Brown et al. (1994) in 24 cheetah females ranged from 25 to 750 ng/g, where values exceeding 100 ng/g were considered peak concentrations. Wielebnowski and Brown (1998) measured estradiol concentration in 14 cheetah females ranged between 17.9 and 408.1 ng/g. The estradiol concentrations measured in this study were within the ranges reported in both previously mentioned studies (see Table 6.). Similar values were measured in lions (186.0 ± 14.8 ng/g), tigers (128.0 ± 13.1 ng/g) and snow leopards (139.7 ± 15.9 ng/g) (Graham et al., 1995).

The reason why the overall level of estradiol was higher in Abiba in comparison to other two females might be (a) higher age, thus possibly higher fertility (Laurenson et al., 1992), and/or (b) different housing conditions, as Abiba was housed in a separate off-exhibit area of the zoo without major disturbance, which is one of favourable conditions (Laurenson, 1993).

The variability of the estradiol concentrations among the three studied females reflects the differences in ovarian cycle dynamics described by Brown and Wielebnowski (1996).

The elevation of the estradiol concentration after the stimulation appeared in all females, and it started declining again after some time (approximately 9 days). The observed increase in the estradiol concentration after the stimulation was considerably lower in Binty in comparison to the other two studied females, suggesting partially supressed ovarian activity due to the presence of her cubs. The elevated level of progesterone in Binty during the first two weeks after the stimulation is probably also related to this fact. There are two possible explanations for the apparent increase of estradiol concentration in Abiba about a day before the stimulation: (1) the estradiol level started increasing due to a natural estrus of the female, or (2) there was a random fluctulation in the estradiol concentration which broke out into an estrus due to the stimulation inlfuence.

According to Meltzer (1999), females should not be kept in proximity to males. If this criterium is not fulfilled, females may habituate to the male presence and their libido and breeding success may be descreased. Although Abiba was neighbouring with the resident male, it did not appear to have a negative impact on her ovarian activity.

The concentration of progesterone exhibited slight fluctulations, it was, however, not significantly affected by the stimulation. Progesterone concentrations reported in cheetahs by Czekala et al. (1994) ranged from 8 to 132 ng/g, which is similar to the values measured in this study (see Table 6.). In lions, measured baseline fecal progesterone values were significantly higher (412 ± 126 ng/g) (Umapathy et al.,

2007). A comparison of progesterone concentrations with other felids was not possible, because other authors only reported the values of all progestagens together.

Although it is rare, ovulation after non-coital stimulation by male was reported in some cheetah females (Brown et al., 1996). The low levels of progesterone in this work suggested/suggest that no ovulation occured in any of the three females within the study period. However, in ovulating female cheetahs, the progesterone concentrations increase within 1 to 10 days after the estradiol surge (Brown et al., 1996). Thus, it was not possible to determine whether the ovulation was triggered by the stimulation or not, due to the short time period of the measurements. The observation that mating is required for ovulation to occur was previously reported by Czekala (1994), Wildt et al. (1993) and Bertschinger et al. (1984). The same also apllies to tigers (Seal et al., 1985), pumas (Bonney et al, 1981) and snow leopards (Schmidt et al., 1993). On the other hand, lions (Schmidt et al., 1979), leopards (Schmidt et al., 1988) and clouded leopards (Brown et al., 1995) may occasionally ovulate spontaneously.

It is possible that males use acoustic and chemical communication to induce sexual behaviour in females passing through their territory (Eaton, 1974). It is known that estrogens are responsible for sexual behaviour (Pineda and Dooley, 2003) and it has been proven that some types of behaviour such as rolling, urine spraying and others are correlated with increased levels of estradiol in cheetahs (Wielebnowski and Brown, 1998). Although behavioural analysis was not a goal/aim of this research, interestingly some of the estrus-related sorts of behaviour were expressed by all the studied females shortly after the beginning of the stimulation (rolling in all females, urine spraying in two cases). However, without a proper behavioural analysis, it is not possible to make any conclusions.

Eventhough estradiol and progesterone are transported into faeces through enterohepatic cycle, it was proven that these hormones can be found in faeces in their free form in cheetahs. Other steroidal substances related to the estradiol and progesterone metabolism, however, occur in faeces in higher concentrations. This is in accordance with the general knowledge of other animals (Squires, 2010). Although,

according to Robinson and Williams (1958 in Shille et al., 1990), cats seldom form glucuronides, estradiol conjugates identified in this study include besides sulphates also glucuronids.

The olfactory stimulant was presented to females in a perforated plastic bottle in order to prevent direct contact of animals and the excrement. The usage of a faecal material from non-resident cheetah male(s) was not possible due to the risk of disease transmission. An alternative approach that might solve both problems could include scent transfer on a piece of cloth, similar as is done in training of the police scent-discriminating dogs (Settle et al., 1994; Stockham et al., 2004). The scent transmission proceeded without direct contact of a scent source, and a scent trap would therefore be sterile.

7. Conclusion

My research showed that, in cheetahs, estradiol and progesterone can be found in faeces in their free form, as well as conjugated or unconjugated metabolites. The found metabolites related to estradiol were estriol, estrone, estradiol-3-sulphate, estrone-3-sulphate, estradiol-3-glucuronide and estrone-3-glucuronide. Progesterone related metabolites included 11, 17 and 20-OH-progesterone.

The study revealed that the ovarian activity of a cheetah female can be stimulated by sent and vocalization of a conspecific male, concluded from significantly elevated estradiol concentration during two weeks after the acoustic and olfactory stimulation. Similar stimulation might be put into practice in breeding facilities in order to enhnance a female's receptivity and willingness to mate and so improve their breeding success. Whether this kind of stimulation is sufficient to induce ovulation, which in cheetahs is usually triggered by mating, still needs to be tested.

The usage of a larger number of individuals subjected to the stimulation would be preferable in further research in order to ensure a higher significance of the results. On the top of that, a modification to the olfactory stimulus presentation by using scent transfer techniques might be an interesting and safe improvement of the methodology.

8. References

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