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Reproduction and pregnancy diagnosis in donkeys

Diploma Thesis

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Declaration

I hereby declare that this thesis entitled ,,Reproduction and pregnancy diagnosis in donkeys´´ is my own work and all the sources have been quoted and acknowledged by means of complete references.

In Prague

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Bc. Lucie Hyniová

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I would like to express my sincere thanks to Mrs. Bendová and Mr. Toman who graciously welcomed me on their farms. I would also like to thank Mrs. Fedorova and Mrs. Skálová for their continuous support and academic expertise. Last but not least, I would like to thank my family for supporting me during my university studies.

Abstract

This thesis was written to evaluate three simple pregnancy diagnosis tests in donkeys. The theoretical part was focused on literature review of anatomy, pregnancy, breeding of donkeys, and methods of pregnancy diagnosis. In the practical part, 54 urine samples were collected from 18 pregnant and non-pregnant jennies. Collections took place every 6-9 weeks to cover different stages of pregnancy. Samples were tested immediately after collection for urinalysis. Urinalysis showed average pH and specific gravity, however the values were not influenced by the pregnancy status. For seed germination test, seeds of mung beans (*Vigna radiata*) and winter wheat (*Triticum aestivum*) were tested with urine in dilution 1:4 and 1:14. Number of germinated seeds were counted 24, 48 and 72 hours after onset of the experiments. Lengths of shoots were measured 72 hours after onset of the experiments. For barium chloride test, 5 ml of 1% solution of barium chloride was mixed with 5 ml of pure urine. The Cuboni reaction was carried out in the State Veterinary Institute, Prague. In case of seed germination, wheat seeds with urine concentration 1:14 showed significant relationship between seed germination measured after 72 hours and pregnancy status. In the same seed and concentration, the shoot length was significant to pregnancy status. In both criteria, pregnant females had lower results compared to non-pregnant females. However, the season also influenced the results. No connection between shoot length and pregnancy status was found. Barium chloride test was concluded as not suitable for pregnancy diagnosis in donkeys since high percentage of false-negative results occurred. On the other hand, Cuboni reaction was concluded as applicable to pregnancy diagnosis in second half of pregnancy, where the results were 100 % correct. Nevertheless, non-invasive pregnancy diagnoses in donkeys require more extensive future research.

Key words: urine, Cuboni reaction, seed germination test, barium chloride test, reproductive hormones, *Equus africanus asinus*

Abstrakt

Tato diplomová práce byla napsána, aby zhodnotila tři jednoduché testy pro diagnostiku březosti u oslů. Teoretická část byla zaměřena na přehled dostupné literatury z anatomie, březosti a chovu oslů, a také metod diagnostiky březosti. V praktické části bylo odebráno celkem 54 vzorků moči od osmnácti březích a nebřezích oslic. Odběry probíhaly v intervalu šesti až osmi týdnů, aby byly ve výzkumu zahrnuty různé fáze březosti. U vzorků byl ihned po odběru proveden rozbor. Rozbor moči ukázal průměrné pH a specifickou hmotnost. Tyto hodnoty však nebyly ovlivněny reprodukčním stavem. Pro test klíčivosti semen byla použita semene fazole mungo (*Vigna radiata*) a pšenice ozimé (*Triticum aestivum*). Tato semena byla testována s močí v poměru 1:4 a 1:14. Množství vyklíčených semen bylo sčítáno 24, 48 a 72 hodin od počátku experimentu. Délky klíčku byly změřeny 72 hodin od počátku experimentu. Pro test chloridem barnatým bylo požito 5 ml 1% roztoku chloridu barnatého, který byl smíchán s 5 ml moči. Cuboniho reakce byly provedeny ve Státním veterinárním ústavu Praha. V případě klíčivosti semen byl nalezen signifikantní vztah mezi klíčivostí semen pšenice při koncentraci moči 1:14 měřených po 72 hodinách a reprodukčním stavem. U stejného semene při stejné koncentraci byla délka klíčků signifikantní k reprodukčnímu stav. U obou kritériích byly výsledky březích samic nižší než výsledky nebřezích. Avšak výsledky byly ovlivněny i ročním obdobím. Z důvodu vysokého procenta falešně negativních výsledku byl test chloridem barnatým shledán jako nevhodný pro testování březosti u oslů. Na druhou stranu Cuboniho reakce byla shledána použitelnou k testování ve druhé polovině březosti, kde byly výsledky ve sto procentech případů správné. Nicméně v budoucnu bude zapotřebí dalšího výzkumu neinvazivních testů březosti u oslů.

Klíčová slova: urine, Cuboni reaction, seed germination test, barium chloride test, reproductive hormones, *Equus africanus asinus*

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List of Abbreviations

ABS	appetitive sexual behavior
AI	artificial insemination
BaCl2	barium chloride
BCS	body condition scoring
CL	corpus luteum
CSB	consummator sexual behavior
DSAFP	Department of Animal Science and Food Processing
eCG	equine chorionic gonadotropin
ET	embryo transfer
FAO	Food and Agricultural Organization
FH	foal-heat
FSH	follicle stimulating hormone
GL	gestation length
GnRH	gonadotropin releasing hormone
HCL	hydrochloric acid
IVF	in vitro fertilization
LH	luteinizing hormone
PPe	postpartum estrus
PR	pregnancy rate
RTU	real time ultrasonography
SE	standard error
SVI	State Veterinary Institute

1. Introduction

Donkeys, or so called domestic asses (*Equus asinus*), play a big role in many cultures (Bohra and Bahura, 2013). The name donkey comes from word 'dunkey' which meant grayish-brown color in the old English language (Hagstrom, 2004). Donkeys are mainly used for transportation of people and possessions (Starkey and Starkey, 1997; Albano et al., 2004; Pritchard et al., 2005; Salimei, 2011; Den Boon, 2012). Usage of donkeys may be divided on the basis of level of development. In developed countries, donkeys are kept as pets, companions for other farm animals, guarding sheep herds or for shows. On the other hand, in developing countries having a donkey only as a pet would be inconceivable (Starkey and Starkey, 1997; Pugh, 2002; Carluccio et al., 2008; Aronoff, 2010; Trawford, 2011). In poor agricultures, donkeys are applied for pulling, threshing, raising water or milling. Children are very often manipulating with donkeys (Starkey and Starkey, 1997). According to study realized in Afghanistan, Egypt, India, Jordan and Pakistan, most of the owners do not treat their donkeys well. 70 % of studied donkeys were thin or very thin and had many wounds and diseases since there is no welfare (Pritchard et al., 2005). During 5 years study when 5481 donkeys, 4504 horses and 858 mules were evaluated in nine developing countries it was found out, that more than 90 % of equids had unhealthy hoofs and limbs and only 15 % of equids were not suffering from undernourishment. These deficiencies were more often noticed in old equids which lived in warmer climate (Burn et al., 2010). According to welfare research from Jordan, out of 86 sampled donkeys 20 % of donkeys were classified as thin. Many donkeys had quite unhealthy extremities. 10 % of donkeys did not have standard gait and 69 % had swollen flexor tendons and fetlock joints (Whay et al., 2006).

In some developing countries, donkeys are also important as a source of milk. Donkey´s milk is a suitable alternative of cow's milk for people who are intolerant to cow´s milk proteins. The disadvantage of donkey's milk is a lower fat content compare to cow´s milk (Carluccio et al., 2008; Salimei, 2011; Contri et al., 2014). Breeding of donkeys for meat is considered very rare. Nevertheless even in one European country (Italy), there are still donkey meat farms (Starkey and Starkey, 1997). Another important utilization of donkeys is crossbreeding to produce mules or hinnies (Pugh, 2002; Carluccio et al., 2008). If mare is mated by a jack, the result is a mule. Mules are very popular in US as ridding and pack animal (Hagstrom, 2004). To get a hinny, jenny is mated by a stallion. It is important to highlight that both crossbreeds are not fertile because

of the incompatibility of the maternal and paternal chromosomes. Nevertheless, the crossbreed females do have an estrus. In 2003 in Morocco, there was a documented case of birth of a male foal to a female mule. Blood samples of this foal were investigated at Cambridge University. DNA analysis of the foal showed occurrence of horse and donkey alleles, so the birth of a male foal to a female mule was considered as true (Kay et al., 2006). Globally, mules are more common than hinnies (Boeta and Zarco, 2005). These hybrids have 63 chromosomes (Kay et al., 2006).

In developing countries, the donkey owners have very poor veterinary support and ultra-sound diagnosis is unreachable. Even for experienced owner, it is not easy to find out the pregnancy of a jenny (Toman, 2014). That is why, in my opinion, non-invasive pregnancy diagnosis has great potential. Levels of reproductive hormone can be detected in different body liquids, such as urine, saliva, blood and also feces (Fickes, 2007).

2. Aims of thesis

This thesis was written to find out if three non-invasive pregnancy tests are suitable for quick, cheap and easy pregnancy testing from urine. The three tests described in methodology are typical for their low price and quite fast results. We can also consider this method as cheap and easy to carry out.

Aim of this thesis was to test three non-invasive methods for quick, cheap and easy pregnancy diagnosis in donkeys from urine.

2.1 Hypotheses

- H1: The urine of pregnant jennies will inhibit germination of mung beans and winter wheat seeds and shoot length more than the urine of non-pregnant jennies.
- H2: The urine of pregnant jennies mixed together with 1% solution of $BaCl_2$ will not coagulate.
- H3: The urine of pregnant jennies will show green opalescence after the Cuboni reaction.

3. Literature review

3.1 General information about donkeys

Donkeys have wide range of height, from miniature donkeys with 90 cm at the withers to biggest donkey breeds, which have around 140 cm at the withers (Pugh, 2002; Hagstrom, 2004). Some experts divide donkeys into three groups: small standards 90-100 cm, standards with 100-120 cm, and large standards with 120-140 cm at the withers (Hagstrom, 2004).

Some people consider donkeys as only smaller prototype of horses. The differences between donkeys and horses are visible not only in the anatomy but also in the physiology and ethology (Burnham, 2002; Hagstrom, 2004; Trawford, 2011; Den Boon, 2012). As an example, there is a different number of chromosomes, donkeys have 62 chromosomes, horses have two more (Hagstrom, 2004).

From their natural environment, donkeys are physiologically used to low energy feed with high level of fiber. Donkeys are considered browsers. Their digestive tract is developed to digest very poor feed and fully use the digestibility potential. The nutrition requirements of donkeys are very modest compare to horses. Therefore there are numbers of obese donkeys suffering from many diseases (Burnham, 2002; Trawford, 2011; Den Boon, 2012). There are two methods to determine body fat content in donkeys. First is standard body condition scoring (BCS) and the second, more innovative one, is real time ultrasonography (RTU). RTU can be used not only for obese donkeys but also for finding body fat reserves (Quaresma et al., 2013).

Compare to horses, donkeys can survive better in hot and dry climate. Donkeys are able to bear quite high dehydration. When they get to water, they drink 25-30 liters of water in a very short time (Den Boon, 2012). Donkeys live longer than horses, their life expectancy is 45 years (Burnham, 2002).

3.2 History and geographical distribution

The domestication of donkeys happened at the same time as domestication of horses approximately 5,000 years ago (Albano et al., 2004; Rossel et al., 2008; Aronoff, 2010; Den Boon, 2012). This period seems to be quite exact but the location of domestication is still very unsure. Based on DNA analysis, scientists assume the African wild asses are the progenitors of all domestic asses (Albano et al., 2004; Den Boon, 2012). The domestication was consequence

of human tendency to colonize and the need for some animal to carry people and their possessions (Rossel et al., 2008). The industry and motorization in developing countries are progressing quite fast and it negatively effects donkeys´ population. This trend is continuing dramatically and we found many donkey breeds endangered (Carluccio et al., 2008; Aronoff, 2010; Veronesi et al., 2010; Gloria et al., 2011; Contri et al., 2014). There are several breeds which will disappear in near future (Carluccio et al., 2008). One of the endangered donkey breeds is Martina Franca donkey (Tosi et al., 2013; Contri et al., 2014). There are only 48 jacks admitted for breeding and 515 jennies left (Contri et al., 2014). Another endangered donkey breed is the Catalonian donkey breed from Spain, with less than 100 jennies (Folch and Jordana, 1997). According to FAOSTAT data from 2013, there are approximately 44.5 million donkeys in the world (Fig. 1). From this amount, 44.5 % donkeys are in Africa, 38.7 % in Asia, 15.5. % in Americas and only 1.2 % in Europe. Countries with highest number of donkeys are Ethiopia, China, Pakistan, and Egypt (Starkey and Starkey, 1997; FAO, 2013).

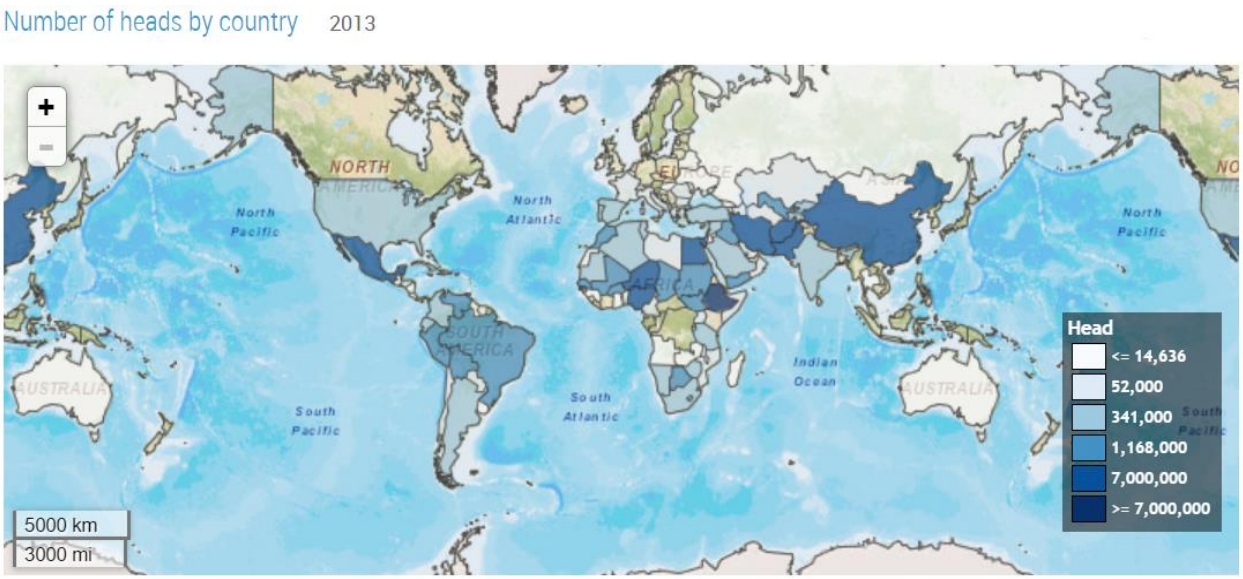


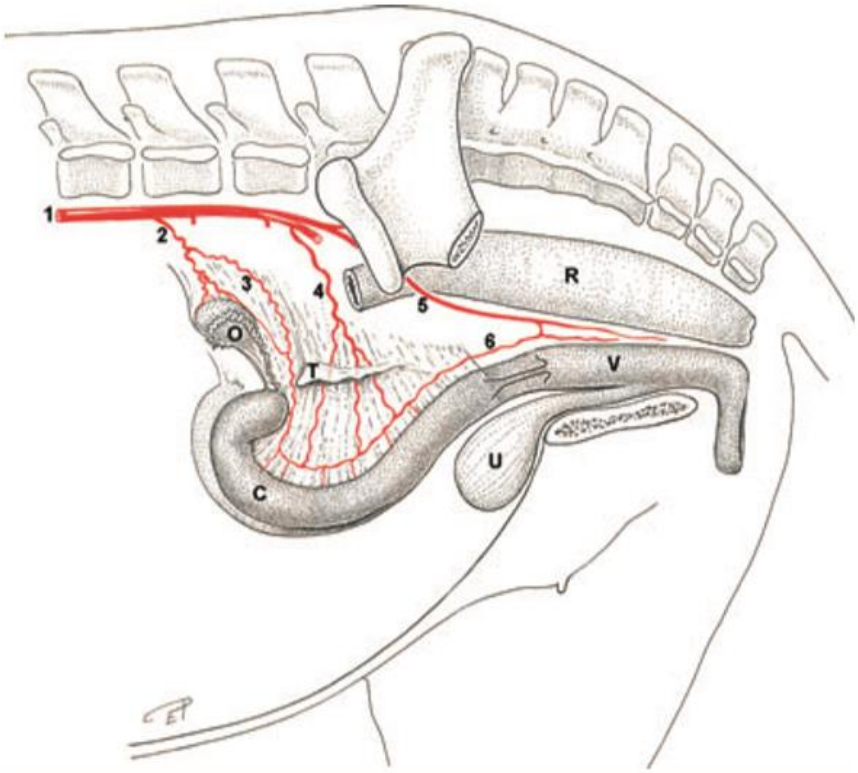
Figure 1: Graphical distribution of donkeys in the world (source: Faostat, 2013)

3.3 Reproduction in donkeys

3.3.1 Anatomy and physiology

3.3.1.1 Jennies

The ovaries have shape of beans, 60–88 mm long and 30–40 mm thick with weight 70-80 g (Purdy, 2005a; Aziz et al., 2008; Renner-Martin et al., 2008). Jennies have Y-shape uterus with two horns of cylindrical shape and each length 250 mm. Other parts of uterus are the neck and



Left lateral aspect of the genital tract in a jenny. 1, aorta abdominalis; 2, arteria ovarica; 3, ramus uterinus of arteria ovarica; 4, arteria uterina; 5, arteria pudenda interna; 6, ramus uterinus of arteria vaginalis; O, left ovary; C, cornu uteri sinistrum; T, ligamentum teres uteri; U, urinary bladder; V, vagina; R, rectum.

Figure 2: Genital tract in a jenny (author: Renner-Martin et al. (2008))

the body (Fig. 2). The uterine body is in abdominal and interfere in pelvic cavity. Uterus main blood supply is arranged by the arteria uterine. Uterine body and vagina are linked by the cervix (50–75 mm in length). The vagina is 150–200 mm long and 100–120 mm in diameter. Vulva with clitoris is external part of vagina and it is bordered by two prominent lips. The reproductive organs fixed to the dorsolateral wall by a complex ligamentous structure. The uterus including the ligaments weighs 0.72–1.61 kg according to study from Romania (Renner-

Martin et al., 2008). If jenny is ovulating, it is possible to find Graafian follicles measure 10-40 mm diameters and corpus luteum (15-35 mm in diameters) (Vandeplassche et al., 1981; Renner-Martin et al., 2008).

Table 1 **Median values and range of maternal, fetal and placental parameters measured in the three groups (pony, Thoroughbred and donkey).**

Variable	Pony (n=7)	Thoroughbred (n=7)	Donkey (n=6)	Steel-Dwass post-hoc
Gestation (days)	327[a] (313–333)	341[b] (324–348)	371[c] (352–376)	[a,b]P < 0.05 [a,c]P < 0.01 [b,c]P < 0.01
Maternal weight (kg)	306[a] (264–326)	588[b] (533–683)	282[a] (256–310)	[a,b]P < 0.01
Maternal age (y)	5 (4–8)	7 (4–12)	6.5 (5–10)	Ns
Parity (n)	2 (1–3)	2 (1–4)	2 (1–4)	Ns
Foal birth weight (kg)	23[a] (20–30)	50.5[b] (46–63)	25[a] (22–31)	[a,b]P < 0.01
Mass of allantochorion (kg)	1.53 (1.4–2.3)	3.5[b] (3–5)	1.39[a] (1.14–1.7)	[a,b]P < 0.01
Gross area of allantochorion (cm2)	7900[a] (7100–9800)	12880[b] (11700–14000)	9300[a] (8600–11300)	[a,b]P < 0.01
Volume of allantochorion (L)	1.70[a] (1.38–2.20)	3.58[b] (2.80–4.0)	1.41[a] (1.06–1.67)	[a,b]P < 0.01
Thickness of chorion (mm)	1.95[a] (1.74–2.72)	2.69[a] (2.29–3.13)	1.42[b] (1.20–1.52)	[a,b]P < 0.01
Volume of chorion (Vc; cm3)	672.3[a] (475.6–772.5)	1019[b] (925.7–1670)	568.6[a] (410.9–842.8)	[a,b]P < 0.01
Microcotyledon surface density (Sv; μm^{-1})	0.03[a] (0.03–0.03)	0.04[b] (0.03–0.04)	0.05[c] (0.05–0.05)	[a,b,c]P < 0.01
Total microscopic area of fetomaternal contact (Sv x Vc; m^2)	18.82[a] (13.56–23.45)	38.46[b] (33.51–66.14)	28.52[c] (20.56–44.04)	[a,b,c]P < 0.05
Rv (Sv x Vc/gross area; m^2)	21.15[a] (17.66–28.8)	29.81[b] (23.94–52.08)	30.18[b] (23.36–39.32)	[a,b]P < 0.05
Placenta efficiency (Foal weight per m^2 placenta; kg $(m^2)^{-1}$)	1.328[a] (1.033–1.622)	1.324[a] (0.937–1.496)	0.873[b] (0.704–1.094)	[a,b]P < 0.01

Different superscripts within rows ([a,b,c]) indicate significant differences found among ponies, Thoroughbreds, and donkeys.

Table 1: source: Veronesi et al. (2010)

Healthy and functional placenta is indispensable for healthy offspring. It was noticed that structurally and functionally normal placenta positively influences the birth of a healthy offspring (Carluccio et al., 2008; Veronesi et al., 2010). If the placenta is not working well, pregnancy complications, such as embryonic or fetal death and intrauterine growth restriction of the fetus, may occur. Comparing to horse or pony, allantochorion of jennies has more complex microcotyledons (all compared parameters are shown in Table 1). These differences

may be related to longer gestation length (Carluccio et al., 2008). It was proved that age is negatively effecting the quality of placenta (Veronesi et al., 2010). The weight of placenta represents 12 % of foal birth weight (Carluccio et al., 2008).

Hormones are essential for well-functioning reproductive system. Gonadotropin releasing hormone (GnRH) is managing to control the influence of all reproductive hormones. Luteinizing hormone (LH) with follicle stimulating hormone (FSH) are needed for development and maintenance of the corpus luteum (CL) and follicular maturation and ovulation (Mottershead, 2001). On the opposite is prostaglandin which negatively effects the lifespan of corpus luteum. Degradation of CL leads to onset the estrus. $PGF_2\alpha$, progesterone, progestin and estradiol are synchronizing estrus (Blanchard et al., 1999). Estradiol effects behavior during estrus and also relaxes the cervix (Mottershead, 2001). Levels of main hormones are shown in Fig. 3.

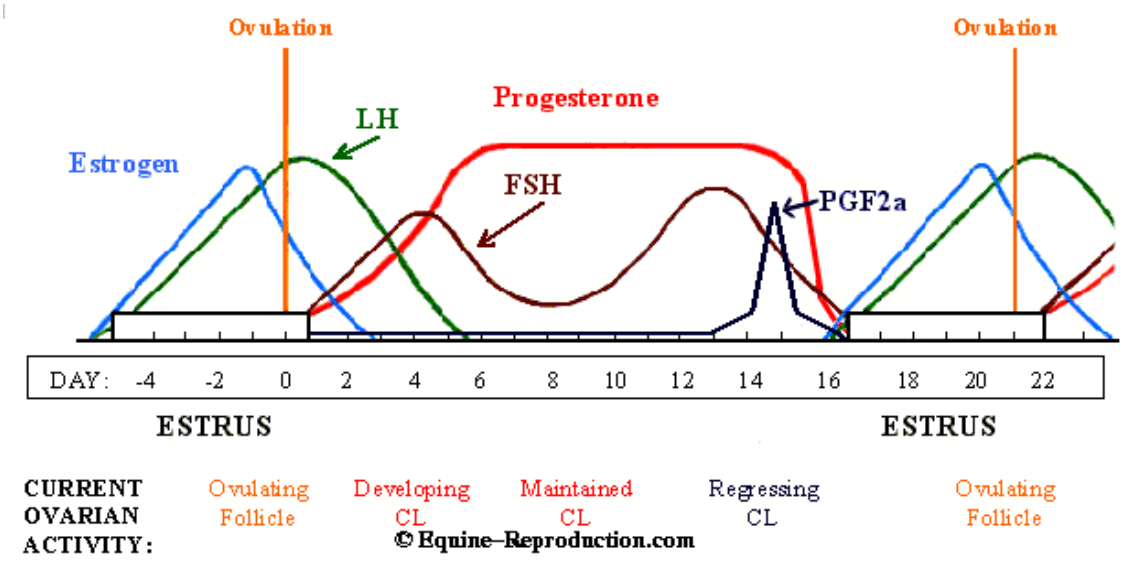


Figure 3: Levels of main hormones in equids (source: Motteshead (2001))

3.3.1.2 Jacks

Jack and stallion have similar reproductive anatomy. One of the difference is in accessory sex glands. The ampulla in jack is larger than in stallion (Pugh, 2002; Purdy, 2005a).

Testicles are hidden in scrotum and oriented vertically (Purdy, 2005a). In the study that compared jack´s testicular volume, it was found no significant differences between the right

and left testes. The testicular volume was measured from 250 to 500 mm^3 (Quartuccio et al., 2011). Penis of jack is 35.5 – 46 cm long when fully erected (Purdy, 2005a). Compare to horses, ration of body size to jacks testicles (Pugh, 2002; Hagstrom, 2004; Purdy, 2005a; Den Boon, 2012) and penis (Hagstrom, 2004; Purdy, 2005a) is relatively large. The size of testicles causes problem during castration of jacks. There is risk of increased blood lose since the donkey has large blood vessels supplying the testicles. Generally, veterinary surgeon do not recommend to castrate in standing position (Hagstrom, 2004).

3.3.2 Reproductive parameters

3.3.2.1 Jennies

Jennies and mares almost do not differ in reproductive anatomy (Pugh, 2002). On the other hand, there are differences in almost every parameters comparing domestic donkey and wild asses. Wild asses are much more seasonal in every reproduction factor comparing to domestic and feral donkeys (McDonnell, 1998) but horses are even more seasonal (Contri et al., 2014). There was found that only 40% of the monitored jennies showed a seasonal anestrus and in the jack the effect of the season is also limited (Contri et al., 2014).

First estrus in jennies usually appears at 8 - 12 months (Purdy, 2005a). The estrus in wild asses usually occurs in spring or early summer, possibly related to climate differences (FAO, 1994; Galisteo and Perez-Marin, 2010) but the effect of environmental conditions (temperature and natural lighting) on estrous cycle was still not completely investigated (Contri et al., 2014). Even in domestic asses is estrus influenced by season (Table 2). Domestic jennies come into the estrus in general every 20-40 days (Blanchard et al., 1999; Pugh, 2002; Hagstrom, 2004; Purdy, 2005b, a; The Donkey Sanctuary, 2013; Tosi et al., 2013). The estrus usually lasts 6-9 days (Vandeplassche et al., 1981; FAO, 1994; Blanchard et al., 1999; Pugh, 2002; Hagstrom, 2004; Galisteo and Perez-Marin, 2010; Tosi et al., 2013). Duration of estrus in jennies is similar to mares (Blanchard et al., 1999). The last two days of estrus are the best period for mating because ovulation comes 5–6 days after the onset of estrus (FAO, 1994; Blanchard et al., 1999; Pugh, 2002; Hagstrom, 2004; Tosi et al., 2013). Follicles in ovulation should be bigger than 25–30 mm. As in horses, there are differences in ovulation across the donkey breeds (Pugh, 2002). In giant breeds, jennies was described multiple ovulation more recently comparing standard size breeds jennies (Pugh, 2002; Hagstrom, 2004). Multiple ovulation leads to higher frequency of twins. Unfortunately like in mares, twin pregnancy is quite risky for the jenny and it is

recommended to eliminate one of the embryos. Nevertheless, this procedure is not easy for the veterinary surgeon so this essay may result to abortion (Hagstrom, 2004).

Table 2 Mean (± SD) duration of estrus, length of diestrus, and length of estrous cycle in 33 mammoth asses

Season	n	Duration of estrus (days)	n	Diestrus length (days)	n	Estrous cycle length (days)
Jan-Mar	22	6.4 ± 2.1	11	16.1 ± 3.5	9	23.3 ± 3.2
Apr-Jun	24	5.3 ± 1.8	16	17.8 ± 2.0	15	23.0 ± 2.8
July-Sept	4	7.0 ± 1.0	3	15.0 ± 1.0	3	22.0 ± 1.0
Oct-Dec	31	5.8 ± 2.4	13	18.6 ± 1.9	12	24.1 ± 2.2
All	81	5.9 ± 2.1	43	17.4 ± 2.6	39	23.3 ± 2.6

Source: Blanchard et al. (1999)

Jennies are polyestral (Henry et al., 1998; Contri et al., 2010; Galisteo and Perez-Marin, 2010) but in study from Spain, the estrous cycle was detected during the year and even there were no differences in the estrous cycle length among seasons (Contri et al., 2014). But on the other hand, there was a significant increase of estrous length in spring and summer compared with autumn and winter (Pugh, 2002; Contri et al., 2014). Dietrus length approx. 19 days (Vandeplassche et al., 1981). Diestrus was shorter in summer than in the other seasons. If we compare jenny and mare again, diestrus is generally longer in jennies (Blanchard et al., 1999). The first heat usually onset around 1 year old jenny and the estrus lasts 2-7 days (FAO, 1994; McDonnell, 1998). In reproductive cycle we distinguish the short-day anovulatory (nonovulatory) season with lasts around 165 days. The long-day ovulatory season is approximately 200 days. The interovulatory interval is 23-25 days (Henry et al., 1998; McDonnell, 1998; Contri et al., 2010; Galisteo and Perez-Marin, 2010). There was found no difference in sexual behavior between ovulatory and anovulatory estrus (Henry et al., 1998).

The gestation length (GL) is 327-380 days according to different studies (Hagstrom, 2004; Carluccio et al., 2008; Veronesi et al., 2010; Tosi et al., 2013; Crisci et al., 2014). According to Galisteo and Perez-Marin (2010) the GL is not influenced by breed, age, sex of the foal or season of breeding, however Hoffmann et al. (2014) found significant different in GL comparing various height categories of donkeys. The miniature donkeys have the shortest GL (mean 357 days), on the other hand the standard size donkeys have GL approximately 385 days. In the middle are the large (giant) with mean 376 days. (Hoffmann et al., 2014) also described

negative correlation to the age of the jenny and that is against the results of (Galisteo and Perez-Marin, 2010). In Table 3 shows comparison of gestation length pony, thoroughbred and donkey.

Table 3 Gestation length

	Mean Gestational Length	Gestational Range
Pony	330	320 - 345
Thoroughbred	340	320 - 360
Donkey	370	360 - 380

Source: Aronoff, (2010)

3.3.2.2 Jacks

If describing jack´s reproductive parameters, we describe mainly about sperm parameters. During ejaculation, which takes 6-12 s, jack ejaculates 10-80 ml of semen. There might be some differences in these parameters in winter because of seasonality of donkeys (Pugh, 2002).

Another sperm parameters which can be evaluated are following (Miró et al., 2013):

- Sperm concentration 273.26×106 ml^{-1}
- pH 7.8
- Sperm viability 77.19 %
- Sperm immature tail 10.03 %
- Sperm coiled-tail 1.36 %
- Sperm head abnormality 2.36 %
- Tailless spermatozoon 2.26 %

In studies in Andalusia and Pêga donkeys (jacks), the research lead to evaluation of following parameters of semen: gel-free volume (36-160 ml), pH (6.8-7.6), sperm concentration (121-531 x 10^6 spermatozoa/ml), motility (71.5-98.9 %) (Canisso et al., 2010; Dorado et al., 2013). This parameters were set up to find out their relation with fertility. The fertility was classified according to pregnancy rate (PR) – fertile (PR more than 60 %), sub-fertile (minimal PR 40 %). The most notable were differences in sperm motility so this factor may be considered as decisive for evaluation of fertility (Dorado et al., 2013). Comparing horse with Catalonian and Martina Franca donkey, the sperm of jacks was more rapid than the stallion sperm and the semen quality of donkeys was excellent (Miró et al., 2005; Gloria et al., 2011). Because of high

concentration of donkey semen, it has satisfactory fertility and this aspect is appreciated in artificial insemination (Purdy, 2005a).

3.3.3 Sexual behavior

Donkey's sexual behavior is strongly affected by domestication. In wild asses, the behavior is more intensive and unyielding. Behavior is not depended only on domestication but also on season, temperature, migration, area of territory and number of animals in herd (McDonnell, 1998; Canisso and McDonnell, 2010; Contri et al., 2014).

3.3.3.1 Jennies

Many estrus sings of jennies were observed. Rhythmic eversion of the vulvar labia (winking) with exhibition of the clitoris, recent urination, male receptivity, clapping, neck and head extension, tail raising, ears back against the neck (Vandeplassche et al., 1981; Henry et al., 1998; McDonnell, 1998; Pugh, 2002; Hagstrom, 2004; Purdy, 2005b, a; Canisso and McDonnell, 2010; Contri et al., 2014). Jenny is more active comparing with mares. Jenny is targeted strait to jack, she may even chase after him. This behavior occurs less frequently as the level of domestication increases (Canisso and McDonnell, 2010). We may observe also behavior familiar in cows and that is mounting of two females. This mounting leads to stimulations of the jack (Hagstrom, 2004; Canisso and McDonnell, 2010). In some cases, there is no need of visual contact of a jenny and jack to start the estrus signs. The vocalization of the male is sometimes enough. Silent estrus with quiet ovulation occurs in low intensity. If the jenny do not accept the present of the jack, she react negatively by moving back, swishing the tail, holding her ears back, clamping the tail against the perineum, kicking with the hind legs (Henry et al., 1998). During diestrus jenny responds negative to mating attempts of jack. Jenny is running or turning away or strongly kicks or bites (Henry et al., 1998; McDonnell, 1998).

Especially in wild jennies, there were observed intensive protection behavior of their young ones and they are able to make a mountain lion or tiger run away because of strong kicking (Burnham, 2002). Their maternal instincts are very strong and also domestic jennies protect vigorously their offspring (Hagstrom, 2004). The domestic jennies use their hind legs for kicking, too. Interesting is that jacks use the front leg for same reason. As pets, jennies sometimes get problematic and difficult to handle during estrus but the hormones do not affect their behavior as intensively as in jacks (The Donkey Sanctuary, 2013).

3.3.3.2 Jacks

Jacks have also specific behavior in breeding season. They investigate jenny´s excrement and cover them with urine. This behavior is not effected by environment, housing, sociosexual conditions, exposure to breeding, or age (McDonnell, 1998; Canisso et al., 2010; Canisso and McDonnell, 2010; Carluccio et al., 2013). Jacks are territorial, non-harem breeders and they do not have so harem manners as stallions (Canisso and McDonnell, 2010). The female sexual signals seems to be important for the sexual stimulation of the male however the breeding potential is high (Henry et al., 1998; Canisso and McDonnell, 2010).

There are described two types of sexual behavior: appetitive (ASB) and consummator sexual behavior (CSB). ASB contains all spectrum of manners of courtship such which will be described later (vocal and visual stimulation, chemical communication via pheromones) and it is considered as adaptable. CSB is more stereotyped and it is actually next stage of ASB because CSB includes the stimulation of sexual organs until the coitus is reached. To switch from ASB to CSB is required male´s isolation. The isolation is indispensable for activation of hypothalamic-pituitary-gonadal axis. Study from Italy was comparing how long both mentioned types of sexual behavior last and what effects the duration. There was found significant difference in duration between natural and induced breeding season. In induced breeding season, the isolation needed to take longer (Carluccio et al., 2013).

If the mating is natural, jack tease jenny in estrus immediately after jenny enters jack territory. This teasing goes side by side with loud vocal expressing, sniffing and flehmen responding. This continue in mounting without an erection and mouth clapping (Pugh, 2002; Purdy, 2005a; Canisso et al., 2010; Canisso and McDonnell, 2010; Carluccio et al., 2013)**.** The teasing is sometimes connected with (sometimes very intensive) aggression of the jack, especially immediately after first introduce. Biting the neck, back and legs is not unusual. To avoid injuries of the jenny (which are quite common), jack can wear breeding muzzle until he calms down. That generally takes 15-30 minutes (Purdy, 2005a). The mounting without erection generally occurs twice more often in young jacks. When jack is close to achieve the erection, he stops with the teasing for a while and with erection, he starts teasing the jenny again and that is followed by copulation (Pugh, 2002; Canisso and McDonnell, 2010). Chemical communication via pheromones is also essential part of sexual behavior (Purdy, 2005a; Carluccio et al., 2013).

The time to reach the erection is usually longer in jacks (5-30 min.) (Pugh, 2002; Hagstrom, 2004; Purdy, 2005b, a; Canisso et al., 2010) comparing to stallion (10-11 min) (Pugh,

2002; Hagstrom, 2004). Since the erection takes so long in donkeys, there are situation when the jack is trying to cover the jenny for hours but without successful breeding (Hagstrom, 2004). The period from jenny entering the territory to copulation is very individual. In natural mating, it all follow the sexual behavior but in in-hand mating or semen collection, it needs to be controlled by the owner (Canisso and McDonnell, 2010). Jacks might get very aggressive during breeding season so domestic jacks should be kept separately (Burnham, 2002; The Donkey Sanctuary, 2013).

According to (Canisso et al., 2010) differences in sexual behavior depended on age of jacks are proved. The flehmen response frequency was higher in young jacks (3.5 years old) so was the number of mounts without erection. Old jacks (14-16 years) needed half time to reach full erection however the young jacks were faster in time from erection to insertion. The ejaculation took longer to the old jacks (28 sec.) comparing to young jacks (22 sec.).

Sexual hormones may take control over one-year-old jacks and incest mating might occur (with mother or sister). If the inbreeding results to a foal, this foal can be affected by genetic disorders so obviously this inbreeding is unwanted. Jacks older two years of age, can change their behavior and may be difficult to handle and may cause problems to their owners. So if people want to keep donkeys as pets, jacks need to be trained well to be calm and controllable. To avoid all these problems, it is recommended to castrate donkey males between 6 and 18 months of age. If the castration is done to older jack, mostly is has no effect on improving the bad behavior (The Donkey Sanctuary, 2013).

Due to significant differences in jennies and mares behavior, owners need to be experienced if they want to mate jack with mare and it is not only different height of both individuals. In crossbreeding, it is required to take into account lower sexual interest from both individuals. According to study from Brazil, less than 40 % of mares in estrus are interested in mating with jacks (Canisso and McDonnell, 2010). It is unusual that mare seriously injured the jack. For safer mating is recommended to restraint the mare to protect the jack (Hagstrom, 2004). To reach successful mating, it is advised to stimulate jack and mares with sexual counterpart of their own kind. So to stimulate jack, jenny in estrus can be used as stimulator for jack. In mare, stallion may be used. Donkey breeders applicate a strategy to keep young jacks with fillies from weaning until jacks reach the puberty, so in period from 6 months to two years of age. It is necessary to avoid any contact between jacks and jennies. This strategy leads to jacks which are used to mare's sexual behavior and the crossbreeding is more successful.

Interest of jack in mare is kept whole life so it is expected that jack which reach the puberty in company of mares will prefer mares to jennies forever (McDonnell, 1998; Hagstrom, 2004; Canisso and McDonnell, 2010). It is very rare to have a jack which is able to mate both type of females. The only way how to keep jack and be sure that it can be used for both females is artificial insemination (Hagstrom, 2004).

3.3.4 Breeding and parturition

3.3.4.1 Mating

The goal of breeding is to get live foal ideally in 100 % of pregnancies. Obviously, it is impossible to reach absolute success but to make the probability as high as possible. It is quite helpful to use hormonal treatment to time effectively the date for breeding and plan mating according to ovulation (Blanchard et al., 1999). In general, following methods of breeding are considered as the most common. The less demanding way is let to breeding to nature and put jack and jenny into same paddock (Fig. 4) (McDonnell, 1998; Pugh, 2002; Purdy, 2005a; Canisso and McDonnell, 2010). One jack can be with one to ten jennies (Purdy, 2005b, a). Whole breeding happened with any effort of the owner but it might be a chance of injury of both individuals. This method is applicable also in crossbreeding (Canisso and McDonnell, 2010).

Figure 4: Mating of jack and jenny in a paddock (author: Hans Reinhard, www.images.fineartamerica.com)

Universally, equid modern breeding systems are using in-hand natural mating (Fig. 5) which is recommended second day of estrus (Purdy, 2005a). To increase the success of breeding, the jenny might be check by ultrasound to determine ovulation (Purdy, 2005a). In-hand mating has big advantage and that is safety of the animals because animals are under control of the breeders (McDonnell, 1998; Pugh, 2002; Purdy, 2005b, a; Canisso and McDonnell, 2010). Another advantage is high chance to approximate configure date of parturition (Purdy, 2005a; Aronoff, 2010). It is necessary to

reach the erection in the jack (Purdy, 2005a, b; Canisso and McDonnell, 2010). To stimulate the erection, jack can be allowed to mount the jenny even couple times without erection. Then jack is taken back in distance 4-6 meters from jenny. Erection should come in a few moment and then jack is let to mount the jenny again and mate her (McDonnell, 1998; Purdy, 2005a; Canisso and McDonnell, 2010). It is not abnormal if the jenny kicks the jack in his chest however this behavior also increases jack´s excitement. The kicking comes along with jawing motions with the mouth (Purdy, 2005a).

In crossbreeding, if the jack is young or unexperienced, to reach the erection, breeders apply strategy with another jennies in estrus which are put into paddock next to the mare that will be mated. This strategy positively effects jack erection and sexual potential. In-hand mating is used also for semen collection (Canisso and McDonnell, 2010).

In fertility, donkeys are more successful comparing with horses. Average conception rate in jennies is 78% while mares have average 65% (Hagstrom, 2004).

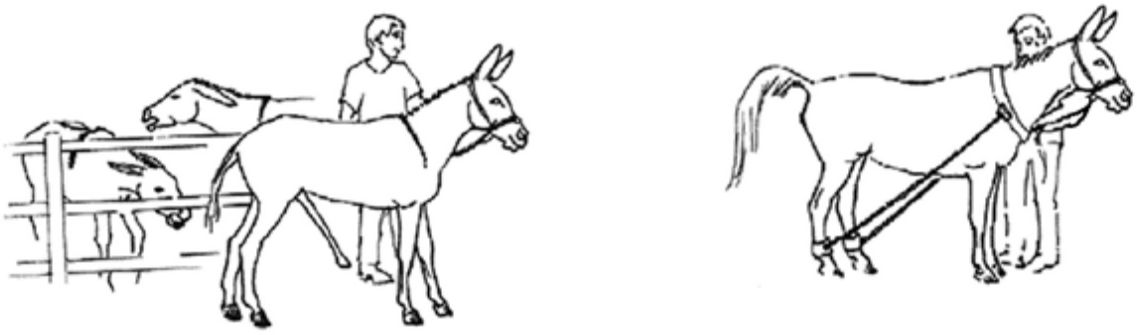


Figure 5: In-hand natural mating (source: Canisso and McDonnell (2010))

3.3.4.2 Pregnancy and parturition

Many changes in hormone levels are happening during pregnancy. From second month of pregnancy, increasing activity of ovaries is registered. Second corpus luteum appears. Concentrations of progesterone is rising in following schema: day 0 (0.9 ng/ml), day 10 (19.9 ng/ml), from day 10 to day 30 is distinguish drop in 12.1 ng/ml. After day 30 to day 40 is increase again at 17 ng/ml. Last phase is decline from day 110 to day 160 (6 ng/ml). Plasma progesterone stays at this level almost to the end of pregnancy, however there is little increase just before parturition. Estradiol concentrations is steadier. Increase is from day 65 to day 165 (1.2 ng/ml). After day 165 is notable decrease (>1 ng/ml), and the lowest concentration is 20 days before parturition (Meira et al., 1998; Hoffmann et al., 2014). Concentration of estrogen is

< 1 ng/ml until day 42. During the middle part of pregnancy the concentration rapidly increases to 600-2700 ng/ml and decreases back to 1-20 ng/ml during the last 2 weeks of pregnancy (Hoffmann et al., 2014).

If a pregnant jenny is watched closely by an owner, the owner may notice that the jenny is becoming solitary and that is one of obvious characteristic of approaching parturition (The Donkey Sanctuary, 2014). Parturition may come any time during day or night, comparing to mares who prefer parturition at night (Aronoff, 2010), nevertheless jennies still give birth in night more often (The Donkey Sanctuary, 2014). The frequency of difficult parturitions is higher in jennies since jennies have a high incidence of cervical adhesions. The owner needs to be prepared for a difficult birth to aid to successful delivery of the foal (Hagstrom, 2004).

Several signs of approaching parturition are described. These signs do not notably differ in jenny and mare. From the external signs are observed enlarging of udder the last months or pregnancy, teats start to swell few days before parturition. It may be also observed softening of pelvic ligaments and elongating of vulva. The closer the parturition, the more nervous the jenny (Aronoff, 2010; The Donkey Sanctuary, 2014). The delivery is quite fast, it may take only 40 minutes from first signs to delivered foal. However, until the placenta in not force out, the parturition is not considered as over (The Donkey Sanctuary, 2014).

The first estrus after delivery, also called foal-heat (FH), appears five to thirteen days after parturition (Hagstrom, 2004; Galisteo and Perez-Marin, 2010; Tosi et al., 2013). Following estrus is called first postpartum estrus (1^{st} PPe) appears approximately 30 days after parturition. Second postpartum estrus (2^{nd} PPe) onsets about 55 days after parturition. It is important to distinguish the estrus since the PR´s differ. In FH the PR is 65.4 %. The highest is in 1^{st} PPe (70.6 %) and lowest is in 2^{nd} PPe (60 %) (Tosi et al., 2013).

In developing countries, the major problem of donkey breeding is mortality of neonates. The reasons are several: jenny in bad condition suffering from shortage of nutrition, poor post-partum care, not full-fledged colostrum. To have good quality colostrum, it is necessary to keep pregnant jenny in good health condition, any illness during pregnancy negatively effects the level of antibodies in colostrum (Aronoff, 2010).

Significant variations are in crossbreeding pregnancies comparing to normal pregnancies. The levels of progesterone and equine chorionic gonadotropin (eCG) are lower in mares inseminated with jack semen. In some tested mares, levels of progesterone and eCG rose in the first two months of pregnancy but then decrease to low level was much faster (day

77) than in mare inseminated with stallion semen (day 126). The biggest problem of crossbreeding is the abortion. The abortion occurrence in mare crossbred pregnancy and normal mare pregnancy was 36.8 % and 21.4 %, respectively. The reasons of aborting are several, however the most recent is the premature luteal regression. Another reason is decrease in concentrations of eCG and progesterone but this reason is considered as indirect since it leads to insufficient creation and premature regression of endometrial cups (Boeta and Zarco, 2005).

Pregnancy rate (PR) is known as it is effected by season and age. Autumn and winter negatively influent the PR. Surprisingly, the PR starts to decrease in jennies very soon, at 6 years of age (Tosi et al., 2013). In a research done in Italy, the scientists transferred vitrified donkey embryos and they compared day 14 and day 25 PR following transfer of donkey and horse vitrified embryos. This study found out that there is a possibility to obtain pregnancies after transfer of vitrified donkey embryos. Comparing the PR´s of donkey and horse, no difference was found. This method could be a good opportunity for long term conservation of endangered donkey breeds (Panzani et al., 2012).

3.3.4.3 Foal

Birth weight is dependent on a breed, however we may consider as a mean 300 kg jenny is having 28 kg foal (Carluccio et al., 2008). Right after delivery, jenny should take interest in the foal and lick it to get it dry. The process is essential to successful bonding. The foal stands up after many unsuccessful attempts and starts to search an udder. Colostrum is indispensable for neonate foal. It is considered as regular if the neonate drinks 1-2 liters of colostrum in first 12 hours of life. If everything goes standardly, a foal is able to move quickly and self-assuredly in 24 hours after delivery (The Donkey Sanctuary, 2014). Because of wide range of gestation length, it might be hard to say if the foal is premature. There are some characteristics that define a premature foal, e.g. weakness, delated standing (more than 2 hours after birth), not intensive suckling reflex, lower birth weight, flopped ears, unstable body temperature, refusing behavior of the jenny or complicated attitude of the jenny (Aronoff, 2010).

3.3.5 Modern technologies in reproduction of donkeys

Because of decreasing population of many donkey breeds, modern technologies are becoming essential for breeding donkeys (Gloria et al., 2011).

Most popular are following methods:

- Artificial insemination (AI)
- In vitro fertilization (IVF)
- Embryo transfer (ET)
- Gamete of embryo cryopreservation

Embryo transfer has been used for conservation of conservation of endangered equid species such as Przewalski's horses (*Equus przewalskii*) but it can be also implicated in donkeys. In a study with five Pantesca jennies, 63 embryos were recovered out of 83 estrous cycles (75.9%) and 98 ovulation (64.3%). Jennies in age 2-5 years old were naturally mated or artificially inseminated with fresh semen (Camillo et al., 2010). In a study with Pega donkeys, the embryo recovery was as successful as in Pantesca donkey. In Pega jennies, the embryo recovery rate was 52.3 %. After the embryo transfer, pregnancy rate was 45.4 % (Peña-Alfaro et al., 2014).

In a research from year 2005, there was compared pregnancy loss in mares mated by jacks or stallions and it was found that pregnancy losses were significantly higher in mares with mule embryo than in mares carrying a horse embryo. There were two groups in this study, the control and the experimental group. In the control group there were observed both embryonic and fetal losses. Most of these losses occurred on day 43 of pregnancy. In the experimental group, all the pregnancy losses were observed after day 40. These losses were classified as fetal deaths and they happened usually day 93 of pregnancy (Boeta and Zarco, 2005).

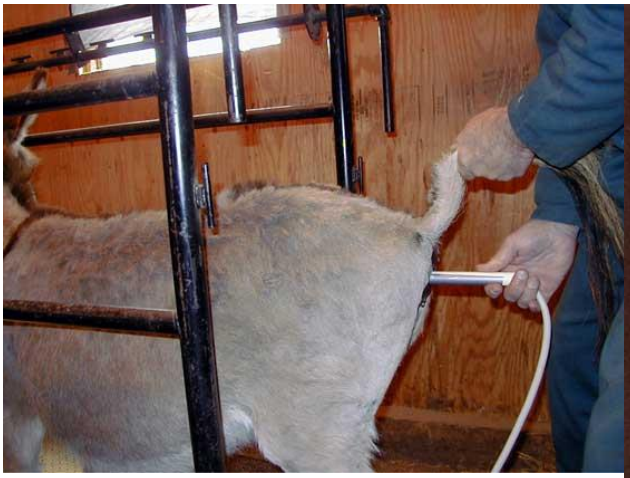
Jacks can be easily trained to ejaculate into artificial vagina (Pugh, 2002; Hagstrom, 2004; Purdy, 2005b). Because of almost similar size of jack´s penis to stallion´s penis, the same artificial vagina can be used for both species (Purdy, 2005a). Semen from artificial vagina is generally frozen or only cooled for artificial insemination (Pugh, 2002; Hagstrom, 2004). The samples may be conserved by skim milk extenders which keep the sperms alive during the transport when the AI is done shortly (Purdy, 2005a). This method is more common in horses but the popularity of usage in donkeys in rapidly growing (Pugh, 2002; Hagstrom, 2004). Nevertheless, jennies cervix is longer than in mares and it makes the AI more complex (Hagstrom, 2004).

Ex situ conservation together with cryopreservation of donkey semen may be applied for endangered breeds. It is import to follow the exact process for freezing the semen to keep it in good condition to get the highest chance for successful fertilization. Jennies have lower probability of fertilization after AI than mares and so it is recommended to add glycerol to

semen before the freezing to prolong the period of storage. But glycerol negatively effects semen fertility and jack´s semen is more sensitive to glycerol. It is necessary to dose glycerol sensitively to find the balance between its positive and negative effects (Vidament et al., 2009; Rota et al., 2012)

3.4 Pregnancy diagnosis´ methods

In general, we can classified methods of pregnancy diagnosis into three categories. There exist visual, clinical and laboratory methods (Purohit, 2010). Visual methods are not recommended because of their insecure results. Visual methods are for example: non return to estrus (owner observes the mated female and if the estrus does not occur, the owner classified the female as pregnant), cocking of the tail (sing visible in camels, many false positive results) (Purohit, 2010). Simple observation of growing belly of the female may be considered as visual method of pregnancy diagnosis. In my own experience, growing belly of a female is not relevant sing of pregnancy since in developing countries donkeys are usually over-fed. Clinical methods are very common in domestic animals. The two main clinical methods are recto genital palpation and ultrasonography (Purdy, 2005a; Purohit, 2010).



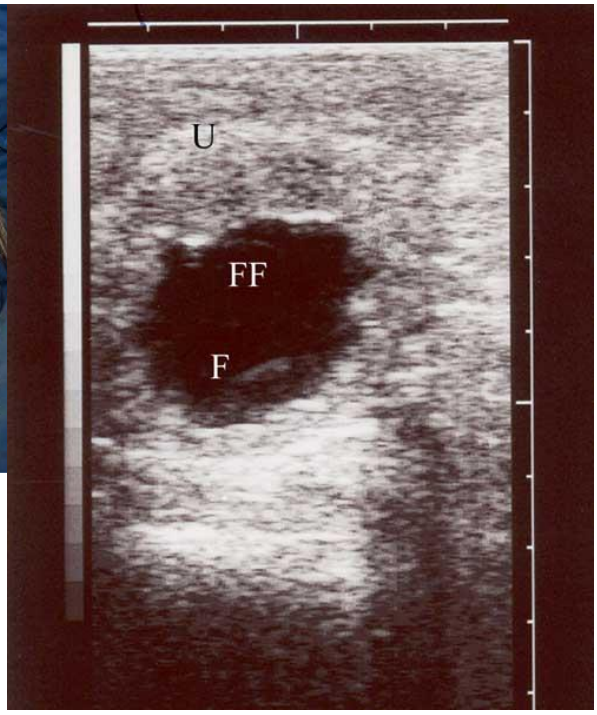


Figure 6: Transrectal ultrasonography (source: Purdy (2005c))

Ultrasound examination is the most common and used on daily basis. Ultrasonography helps to detect not only pregnancy but also to estimate stage of estrus (Purdy, 2005c; Aziz and Lazim, 2012). As mentioned before, the stage of estrus effects determination of the ideal time for AV or hand breeding. In ultrasonography are two basic methods of examination: transrectal

(Fig. 6 showing a 37 day pregnancy, F = fetus; FF = fetal fluids; U = uterine wall) and transabdominal (Fig. 7 showing a 72 day fetus) (Purdy, 2005c). Palpation is most common method in cattle (Broaddus and De Vries, 2005). Laboratory methods are following: Cuboni reaction (test) – described below, assay of gonadotropins – measured from urine and blood plasma from mating to day 90 of gestation by immunoradiometric assays. Gonadotropins assay may be applied only first trimmest since the secretion of eCG peaks 60-80 days of pregnancy. It gives fine results in pregnant equid females and primates (Murphy and Martinuk, 1991; Couture et al., 1993; Hoppen, 1994).

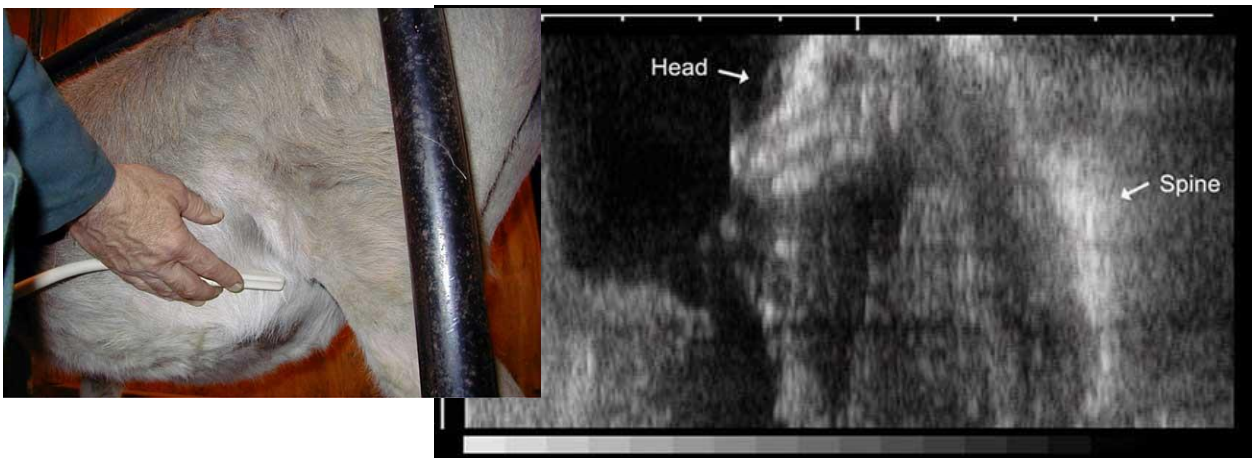


Figure 7: Transabdominal ultrasonography (source: Purdy (2005c)

3.4.1 Non-invasive methods

According to Fickes (2007) urine hormone analysis is considered to be the only exact non-invasive method for detection of hormone levels. However, its negatives are time-demanding and sometimes complicated to provide. Naturally, it is also possible to collect the urine invasively by catheterization or by an endoscope. Invasive methods are uncomfortable for the animals and needs to be performed by a veterinarian (Schumacher and Moll, 2011). Fecal hormone analyses is an alternative to urine hormone analysis since the collection of samples is much easier (Skolimowska et al., 2004; Fickes, 2007).

3.4.1.1 Seed germination test

The basis of seed germination test are dated from ancient Egypt (2100-2200 BC) in papyrii. Test is patterned on the differential response in germination and shoot growth of wheat seeds comparing urine of pregnant and non-pregnant cows (Veena and Narendranath, 1993). There was found repression by the urine of pregnant cows in germination and shoot growth of wheat seeds. In non-pregnant cows, such inhibition was not found (Veena and

Narendranath, 1993; Dilrukshi and Perera, 2009b). Mung beans were also used for testing. The cow urine was diluted with distilled water in ration 1:4, 1:10, 1:14 (urine : distilled water). Measurement of germination was done 24 hours and 48 hours after and the shoot length was measured the 5th day. The abscisic acid is in urine in specific concentration. It has major effect to maintain the dormancy of the seeds (Dilrukshi and Perera, 2009a; Krishna and Veena, 2009). In pregnant cows is higher (170.62 nmol/ml of urine) than in non-pregnant cows (74.46 nmol/ml) (Krishna and Veena, 2009). The pH of urine has no effect on germination of the seeds (Veena and Narendranath, 1993). Seed germination test was successfully used in pregnancy diagnosis in *Bos frontalis.* In this researcher, the scientists used paddy seeds (Perumal, 2014).

In study in Bangladesh, from fifty crossbred cows and heifers, samples of urine were collected early in the morning on day 14, 21, 28, 35 and 45 after artificial insemination (AI) and were used for seed germination test using wheat seeds. Fifteen wheat seeds were put on filter paper into petri dish. The seeds were pour with 15 ml of diluted urine in concentration 1:4. The samples were checked for five days to count the shoot length. The results showed that germination inhibition percentage of AI cows were significantly higher compare to non AI cows and water control groups (Rine et al., 2014). The same procedure was used in Malnad Gidda cattle breed (Narayana Swamy et al., 2010).

Seed germination test was applied in sheep with satisfactory results. There were samples from three pregnant ewes and three non-pregnant ewes. The results showed significant difference between pregnant and non-pregnant groups in germination percentage and shoot length of germinated wheat (Islam, 2013).

In alpacas, the seed germination test was done on mung beans (*Vigna radiata*) and winter wheat seeds (*Triticum aestivum*). Sufficient results were obtained from mung beans treated with urine with 1:4 concentration, nevertheless critical factor was the season which may inaccurate the results (Kubátová et al., 2014).

The seed germination test can be applied in almost every conditions, it is very simple method, however out of the three applied methods it is the most time consuming (Veena and Narendranath, 1993; Krishna and Veena, 2009; Narayana Swamy et al., 2010; Islam, 2013; Perumal, 2014).

3.4.1.2 Barium chloride test

Barium chloride test is quick (3-5 minutes) cheap and easy method designed by Maslov and Smirnov (Ndu et al., 2000). They used 1 % barium chloride solution and pour it into cow urine. If the female was not pregnant, a white precipitate appeared. If the solution was added into pregnant cow urine, the higher level of progesterone and estrogen cause opposite reaction, the solution stays clear without a white precipitate. If the test was performed 15-210 day after artificial insemination, the outcome was 90-100 % correct (Maslov and Smirnov, 1965). This tests are based on chemical reaction of estrogen in urine (Haberová et al., 2011). Cows (heifers) were also tested in Czech Republic. Barium chloride test appeared as quite suitable for pregnancy testing since it showed truly pregnant heifer in 79.7 % cases. However the accuracy in detection of non-pregnant females was 50 % only. In alpacas, the barium chloride test was evaluated as unusable for pregnancy diagnosis (Skálová et al., 2014).

Barium chloride test can be successfully applicate in sows. If the diagnosis is done 39 days after breeding and more, the accuracy of the test in at least 95 % (Ndu et al., 2000). In study from India, the gestation period of sows was divided into three stages. Less than 38 days after breeding, 38 to 76 days and more than 76 days. The correct results were 84 % in 1^{st} stage, 88 % in 2^{nd} and 3^{rd} stages.

Barium chloride test was also examined in 14 females of Bactrian camel. The result were not conclusive as method for pregnancy diagnosis in camels (Haberová et al., 2011). Negative result occurred in 30.77 % non-pregnant females and in 69.23 % pregnant females. The best result in pregnant females were 20 % positive reactions 3rd third of pregnancy (Fedorova et al., in press).

3.4.1.3 Cuboni reaction

It is a simple qualitative method for determining pregnancy based upon fluorescence. This method was invented for testing mares. After heating benzene extracts of acid hydrolyzed urine with concentrated sulfuric acid the green fluorescence in the sulfuric acid phase if it is a pregnant female (Cuboni, 1934). The SVI Prague modified the test because of strong benzene toxicity, benzene was replaced with toluene instead of benzene (Kubátová, 2014). As Barium chloride test, Cuboni test is also based on chemical reaction of estrogen in urine (Cuboni, 1934; Haberová et al., 2011).

Cuboni reaction is that is commonly used for pregnancy diagnosis in horses with satisfaction results (Purohit, 2010). This method was also applied on Bactrian camel. In first study, nine females from Czech zoological gardens were tested. Cuboni reaction was consider as useful method in the second half of gestation (Haberová et al., 2011). The second study continued with 14 females of Bactrian camel. The tested animals were evaluated according the third of gestation. It was assumed that higher stage of pregnancy will bring more accurate results. In camels in 3rd third results were 100 % correct. False negative results were occurring quite often in 1st third of pregnancy (57.1%). The season had significant impact on the results. In autumn was problem with dubious reactions, in winter with false positive results. However winter is breeding season and might had an affect also. To sum up, authors considered applicability for pregnancy in camels especially in summer and autumn for testing in Europe (Fedorova et al., 2013; Fedorova et al., in press).

According to Skálová et al. (2014), Cuboni reaction was considered as not suitable for pregnancy diagnosis in cows nor is for alpacas. There was not found any clear relationship between the reproductive status of females and the results of barium chloride tests. There was also no correlation between results of Cuboni and barium chloride reactions.

3.4.1.4 Fecal estrogen measurement

This method was tested on female equids from zoological gardens and sanctuaries. The samples were taken from following animals: 18 Polish primitive horse (*Equus caballus gmelini s. gmelini silvatica*), 5 Shetland pony (*Equus caballus sp*.), 4 Chapman's zebras (*Equus burchelli antiquorum s. Chapmani*) and 4 domestic donkeys (*Equus asinus asinus*). Collecting of samples was done once or twice every two months. The period of colleting was from April to February. The aim of this study was to find out the potential utilization of radioimmunoassay to analyze the total unconjugated estrogen concentration to diagnose pregnancy. Concentration of fecal estrogen was significantly higher in the second and third trimester of pregnancy comparing to non-pregnant females (Skolimowska et al., 2004).

3.4.1.5 Urinary parameters

The pH of equids ranges from 7 to 9 since the pH around 8.5 is considered as optimal (Harkins et al., 1996; Stämpfli and Carlson, 2001; Robert et al., 2010; Goren et al., 2014). Specific gravity in horse (donkey´s date are unable to find) should not be lower than 1.020 and higher than 1.060. Result lower than 1.020 indicates dehydration (Schumacher and Moll, 2011;

Adam, 2012; Schott, 2012). Standard density in equids is from 1.030 to 1.050. It is not abnormal to find small numbers of red blood cells, white blood cells and bacteria in urine sediment (Schumacher and Moll, 2011). Other urinary parameters such as protein, glucose, ketones, bilirubin, blood, and leukocytes are negative in heathy equid. Only urobilinogen standardly range from negative to weak positive (Parrah J. D. et al., 2013).

4. Methodology

4.1 Literature review

Literature review was based on scientific articles from databases Science Direct, Web of Science and Google Scholar. Article searching was based on following key words and similar: urine, Cuboni reaction, seed germination test, reproductive hormones, *Equus africanus asinus*.

4.2 Experimental animals

Female donkeys used in experiment came from two private farms (Fig. 8) owned by: (1) Mr. Zdeněk Toman, Jistebnice č.p. 3, Ounuz, 391 33 and (2) Mr. Jan Benda and Mrs. Martina Bendová, Kostelec n. Vltavou 51, 398 58. Chosen farms were relatively close to the university (about 60 kilometers). Farms bred donkeys for many years so they had big experiences. Both farms kept donkeys as pet animals and also for breeding and selling of foals. Few animals were also used for ridding yet only for small children. Donkeys in Kostelec farm were kept in stable during nights and stayed outside on the pasture during days. Donkeys in Ounuz had only shelters on pastures so they stayed outside permanently. Another difference between farms was the breeding method. In Kostelec the jack was living solitary until a jenny was in estrus. During estrus jenny was placed in a same paddock with the jack. Sometimes even two jennies in estrus were placed together with the jack for mating. This breeding method was important contribution to final statistical evaluation since the dates of mating were known. In Ounuz, the jack lived in a herd with jennies and the mating could take place any time so the date of mating was usually unknown. All mentioned donkeys were quite well

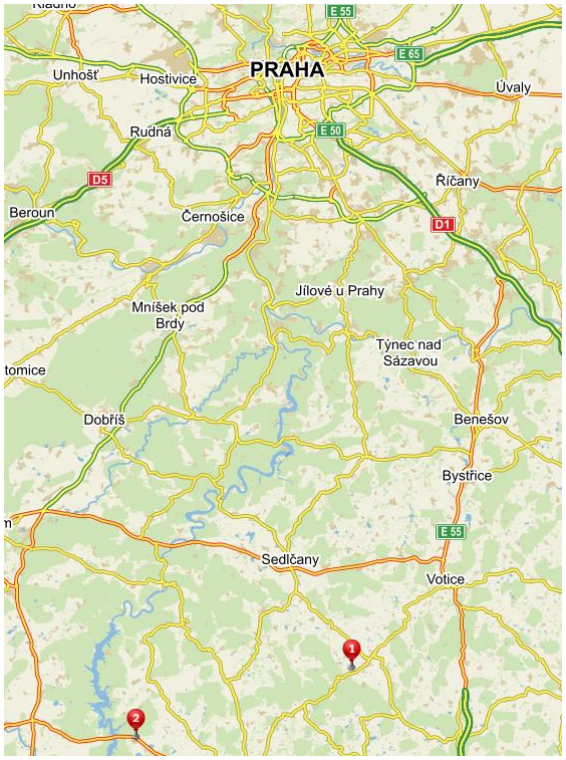


Figure 8: Graphical location of two private farms farm locations (souce: http://www.mapy.cz)

tamed so it was possible to come close enough to get the sample. However, sometimes the collection was impossible according to females' willingness to cooperate as mentioned below. A total of 18 females were included in the research but the number of sampled animals varies due to farm management. I did not test only pregnant females but also non-pregnant for control.

Table 4 Detailed information about tested females

Animal	Farm	Number of collected samples during		Date of last mating	Date of last parturition
		pregnancy	non-pregnancy		
Adélka	Ounuz	3	1	10.10.2013[1]	10.9.2014
Andulka	Ounuz	2	1	23.10.2013[1]	23.9.2014
Kely	Ounuz	0	4	Missing data [2]	2013
Lucinka	Ounuz	3	0	Missing data	26.4.2014
Mína	Ounuz	3	1	Missing data	28.2.2014
Růženka	Ounuz	0	1	N/A (a foal)	N/A (a foal)
Asina	Kostelec	0	5	N/A (a foal)	N/A (a foal)
Aťka	Kostelec	2	0	3.4.2013	12.4.2014
				16.5.2014	Currently unknown
Bubajda	Kostelec	3	0	11.8.2013	6.8.2014
Čuču	Kostelec	2	0	15.5.2014	7.2.2014
Danger	Kostelec	3	0	29.10.2013	28.10.2014
Mala	Kostelec	2	1	8.6.2014	12.10.2012
Melisa	Kostelec	0	2	8.6.2014 [2]	Currently unknown
Princezna	Kostelec	2	0	11.2.2014	6.2.2015
Racina	Kostelec	1	1	22.4.2013	28.4.2014
Rainy	Kostelec	4	0	27.10.2013	1.10.2014
Rozárka	Kostelec	2	0	8.7.2013	15.6.2014
Týna	Kostelec	5	0	19.1.2014	22.1.2015

1 Missing data, only estimation according to the date of parturition

2 Probably infertile

4.3 Collection of samples

The daily routine on farms was not affected by collection of the samples. The jennies were moving freely on their pastures without any capturing or binding. This fact made the collecting more complicated since the pastures were large and hilly. I followed the jennies on

the pasture and waited for the spontaneous urination. Sometimes was not impossible to collect the sample, for example when the jenny had a fresh-born foal and it was not possible to come closer to the jenny since she got solitary and timid for a few weeks. Another problem was the weather. Donkeys almost did not urinate in wet and rainy conditions so that was sometimes an issue, too.

The urine samples were collected at interval 6-9 weeks to cover different stages of pregnancy of tested animals. Urine was collected during spontaneous urination of animals into plastic cups held in hand or fastened to a longer rod (Haberová et al., 2012). The samples were transported in the car fridge to the laboratory of Department of Animal Science and Food Processing (DSAFP) and stored at 5-7 °C for test. Completely were taken 54 samples.

4.4 Basic urinary analysis

Urine was immediately after collection tested on the pasture for biochemical parameters (Reine and Langston, 2005): pH, glucose, bilirubin, ketone, protein, urobilinogen, nitrite, specific gravity, leukocytes - measured by DekaPhan® Leuco test strips (Fig. 9). DekaPhan® Leuco was insert into the urine and after 1 minutes the results were checked according to producer´s instruction (ErbaGroup, 2015). Results from DekaPhan® Leuco were considered as rough since DekaPhan® Leuco was designed from human urine. Despite the fact that DekaPhan® Leuco measures the pH, for more accurate pH was used Duotest® double zone pH-indicator papers according to producer´s instruction (Macherey-Nagel, 2015). Since DekaPhan® Leuco (measurement interval = 1) is less exact that Duotest® double zone pH-indicator papers (measurement interval = 0.3), DekaPhan® Leuco results should be considered more as control measurement. Other measured parameters were density (by dosimeter) and also temperature (by thermometer). Temperature was measure because of its importance for calculation the density formula.

Figure 9: DekaPhan® Leuco test strips (source: http://www.xlab.ro)

4.5 Seed germination test

Seed germination test was the most time-consuming comparing the three tests. Seeds of mung bean (*Vigna radiate*) and winter wheat seeds (*Triticum aestivum*) were bought in quality for human consumption. To make sure that any chemical will not affect the testing, the seeds were in organic quality. Every sample was examined for mung and wheat.

The testing onset was the same day as the sample was collected. For every animal were needed 100 seeds of wheat seeds and 100 mung beans. The seeds were placed in sterile Petri dishes. 50 seeds were counted per every Petri dish. Since the urine was tested in two ratios: 1:4 and 1:14 (urine : distilled water), together were four Petri dishes per one urine sample so for every female there were four different treatments. Prepared seeds in Petri dishes were poured by 20 ml of solution of urine diluted with distilled water in the ratio mentioned before.

Evaluation took place for three successive days after the initiation of the test that means the day of establishing the trial is considered as Day 0. On Day 1, 2, 3; respectively approx. 24 hours, 48 hours and 72 hours after establishing the trial, the seed germination (number of germinated seeds) was counted. Lengths of spouts were measured on Day 3 (approx. 72 hours after establishing the trial). Measuring was in millimeters by a ruler. The results were entered in MS Excel 2013 and later statistically evaluated as mentioned below.

4.6 Cuboni reaction and barium chloride test

The Cuboni reaction could not be done in the laboratory of DSAFP because of manipulation with dangerous chemicals, special equipment for realization and a lot of experiences needed for accurate evaluation of results. The Cuboni reaction was processed in State Veterinary Institute (SVI) Prague. The minimal amount of urine sample needed for Cuboni reaction was 20 ml. Cuboni reaction has following steps: filtration through the filter paper, mixing 5 ml of filtrated urine with 1 ml of concentrated hydrochloric acid (HCl) in the test tube, boiling the test tube water bath for 10 minutes, cooling of the test tube, addition 6 ml of toluene, proper stirring, spontaneous dividing in two layers after 1 min, separation and filtration of the lower layer (contains toluene) into a clear test tube containing 1 ml of concentrated H_2SO_4, stirring and hot water bath (80 °C) again for 15 minutes, cooling, evaluation the color of opalescence in the test tube placed on the dark background (Kubátová, 2014).

Barium chloride test was the third method which was used for pregnancy diagnosis. Barium chloride test was performed in the laboratory of DSAFP since it is a simple test which do not required neither special equipment nor a lot of experiences. Equipment for this test is simple: a test tube (one per every sample), a pipette, 5ml of 1% barium chloride solution, and 5 ml of urine. Urine is poured into a test tube than barium chloride solution is very slowly drip with a pipette. It is recommended to examine the content after 5 minutes (chosen 5 min according to Kubátová et al. (2014) and Fedorova et al. (in press)). Evaluation is done simply by observation – forming the white precipitation (non-pregnant) or clear solution in pregnant female (Fig. 10).

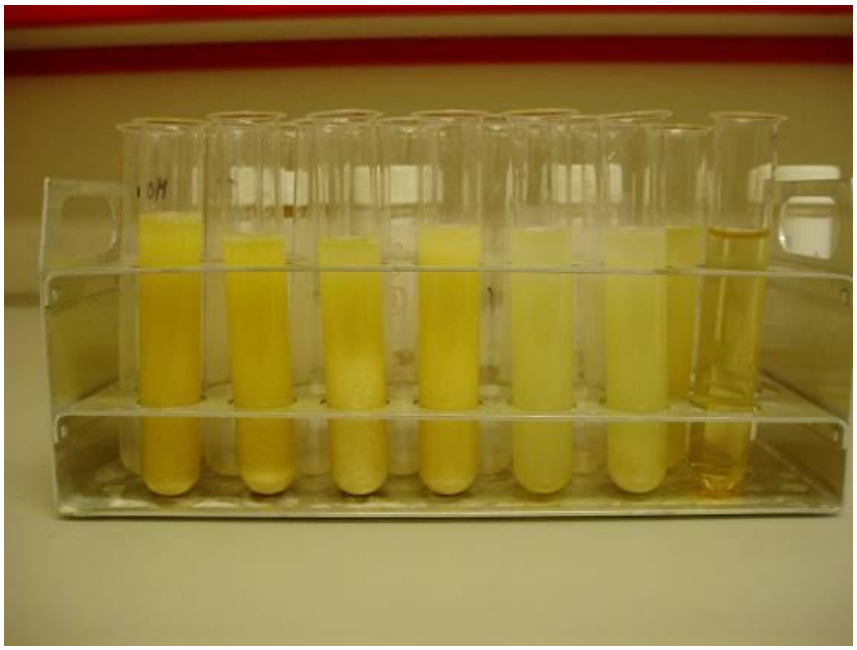


Figure 10: results of a trial of barium chloride test (author: A. Kubátová, 2014)

4.7 Experiments evaluation and data analysis

The results were evaluated according to according pregnancy status (pregnant × non-pregnant) and phases of pregnancy (thirds and halves). Thirds and halves were counted according to standard length of pregnancy (Hagstrom, 2004; Carluccio et al., 2008; Veronesi et al., 2010; Tosi et al., 2013; Crisci et al., 2014). These phases were determined according to day of conception (if it is known) or day of parturition. Due to budget reasons, it was impossible to evaluate the pregnancy by ultrasonography. The actual health status (no visual sings of disease) of animals was also noted. Information about detected reproductive disorders were received from keepers. Another parameter potentially influencing the testing was the season what was also consider in data analysis.

Specific gravity was calculated according density and temperature of the tested urine. Density was measured in 41 samples while they reached temperature of 20 degrees Celsius. The formula for specific gravity is following: $SG = \rho_{substance} / \rho_{H2O}$ (Yesiller et al., 2014).

Data were recorded into the MS Excel. Some simple mathematic procedures such as mean were done in MS Excel. More complicated statistical evaluation was proceed in StatisticaCz 12 program (StatSoft, 2013) in significance level $\alpha = 0.05$. Firstly, it was tested the distribution of data (Kolmogorov-Smirnov test, $p < 0.05$). The data did not have the normal distribution, therefore non-parametric tests were applied. All used non-parametric tests were following: Kruskal-Wallis test, Mann-Whitney U test, Pearson's chi-squared test, and Wilcoxon signed ranks test. The tests used in each analysis are presented in chapter Results. Calculated numerical values with exception of "p value" and specific gravity were rounded off to two decimal places.

5. Results

5.1 Urinalysis

The mean ± SE pH (n = 54) measured by Duotest® double zone pH-indicator papers was 8.77 ± 0.03. The lowest pH measured in 37.03 % samples was 8.5, pH 8.8 in 38.89 %, pH 9.1 in 22.22 %, the highest pH value 9.4 occurred only once (1.85 %). The mean ± SE pH by DekaPhan® Leuco was 8.54 ± 0.07. To be more specific, pH 8 in 46.30 % samples, pH 9 in 53.70 % samples. Significant difference was found between measuring pH by Duotest® double zone pH-indicator papers and DekaPhan® Leuco test strips (Wilcoxon signed ranks test: $T = 428.50$, $Z = 2.70$, $p < 0.001$). Results of pH from DekaPhan® Leuco test strips was significantly lower.

Evaluation of relationship between pH and pregnancy status of the animal showed no statistically significant relationship. The results were not significant neither from Duotest® double zone pH-indicator papers (Mann-Whitney U test: $U = 318.00$, $Z = -0.10$, $p = 0.92$) nor from DekaPhan® Leuco test strips (Mann-Whitney U test: $U = 225.00$, $Z = 1.81$, $p = 0.07$).

When comparing pH among farms, no significant difference occurred Duotest® double zone pH-indicator papers value (Kruskal-Wallis test: $H (1, N = 54) = 2.45$, $p = 0.12$). However, there was significant difference among farms in DekaPhan® Leuco pH value (Kruskal-Wallis test: $H (1, N = 54) = 8.68$, $p < 0.001$).

Measured values of urine density ranged from 1020 kg/m^3 to 1065 kg/m^3. Mean ± SE was 1043.05 ± 1.26 kg/m^3. The mean of specific gravity (n=41) was 1.045 ± 0.001. Both, the lowest value (1.022) and the highest value (1.067) occurred in 2.43% samples. Relationship between specific gravity and pregnancy status of the animal was not statistically significant (Mann-Whitney U test: $U = 162$, $Z = -0.55$, $p = 0.58$). Specific gravity was also measured as a part of DekaPhan® Leuco test strips. Results were following: 1,000 in 12 samples (22.22 %), 1,005 in 41 samples (75.93 %), and 1.05 in one sample (1.85 %).

Specific gravity was also evaluated among farm and there was shown significant difference (Kruskal-Wallis test: H (1, N = 41) = 5.87, p = 0.02), see Figure 11.

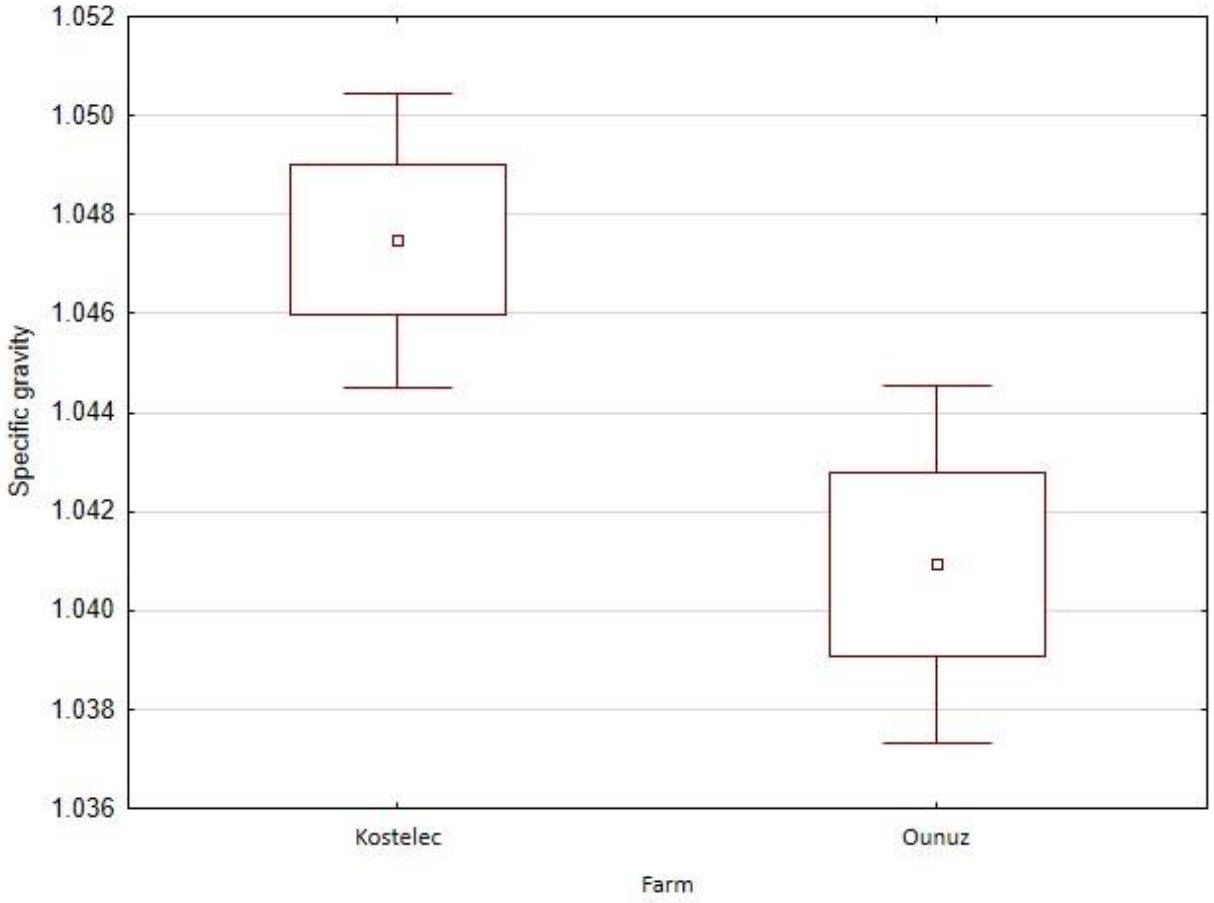


Figure 11: Differences in specific gravity among the farms

Following parameters were measured by DekaPhan® Leuco with negative results in 100 % of samples: leucocytes, nitrites, glucose, ketones, and hemoglobin. Blood was positive in one sample, however in the lowest level (light positive). Protein was presented in 33 samples (61.11 %). Protein reached three levels: 0.3 g/l (30 mg/dl) in 30 samples (90.91 %), 1.0 g/l (100 mg/dl) in one sample (3.03 %), and 5.0 g/l (500 mg/dl) in two samples (6.06 %). Higher level of urobilinogen, 17 µmol/l (1 mg/dl), occurred in 51 samples (94.44 %). The remaining three samples (5.56 %) did not show higher level of urobilinogen. Weakly positive (+) bilirubin was found in 51 samples (94.44 %).

5.2 Cuboni reaction

There was found statistically significant relationship between results of Cuboni reaction and pregnancy status of animals (Pearson Chi-square: 19.70, df = 1, p < 0.001). In samples of pregnant females, 25.00 % were false-negative and 75.00 % positive. In non-pregnant females,

88.89 % samples were negative and 11.11 % were false-positive. Statistically significant relationship also occurred between Cuboni reaction result and thirds of pregnancy, see Table 5 (Pearson Chi-square: 34.32, df = 3, $p < 0.001$) and between Cuboni reaction result and halves of pregnancy (Pearson Chi-square: 26.94, df = 2, $p < 0.001$), see Table 6.

Table 5 The results of the relationship between Cuboni reaction result and thirds of pregnancy

Result	Non-pregnant	1st third of pregnancy	2nd third of pregnancy	3rd third of pregnancy
Negative	88.89 %*	72.73 %	6.67 %	0 %
Positive	11.11 %	27.27 %*	93.33 %*	100 %*
No. of samples	18	11	15	10

Table 6 The results of the relationship between Cuboni reaction result and halves of pregnancy

Result	Non-pregnant	1st half of pregnancy	2nd half of pregnancy
Negative	88.89 %*	45.00 %	0 %
Positive	11.11 %	55.00 %*	100 %*
No. of samples	18	20	16

* An asterisk marks the true results of the test

Statistically significant relationship between season of sample collecting and results of Cuboni reaction was not observed (Pearson Chi-square: 5.17, df = 3, p = 0.16).

According to results from Cuboni reaction, the third hypothesis that the urine of pregnant jennies will show green opalescence after the Cuboni reaction, was accepted.

5.3 Barium chloride test

Evaluation was done according pregnancy status and according different stages of pregnancy. The results of barium chloride test (n = 54) were not significantly affected by the real reproductive status of females (Pearson Chi-square: 1.95, df = 1, p = 0.16). In pregnant females (n = 36), 69.44 % samples were false-negative and 30.56 % were positive. Results for non-pregnant (n = 18) were 50 % negative and 50 % false-positive. There was also not found any statistically significant relationship between results of barium chloride test and halves of pregnancy (Pearson Chi-square: 3.67, df = 2, p = 0.16). Females in 1st half (n = 20) had 60.00% false-negative and 40.00 % positive results. Samples from 2nd halves of pregnancies showed 81.25% false-negative and 18.75 % positive results. The barium chloride test results from samples ordered according thirds of pregnancies are presented in Table 7. Neither in thirds of

pregnancy and barium chloride test results was not find any statistically significant difference (Pearson Chi-square: 2.63, df = 3, p = 0.45).

Table 7 The results of the barium chloride test

Result	Non-pregnant	1st third of pregnancy	2nd third of pregnancy	3rd third of pregnancy
Negative	50.00 %*	63.64 %	66.67 %	80.00 %
Positive	50.00 %	36.36 %*	33.33 %*	20.00 %*
No. of samples	18	11	15	10

* An asterisk marks the true results of the test

Since the sample collection coved all seasons, no effect of season on the results of barium chloride test was found (Pearson Chi-square: 4.18, df = 3, p = 0.24).

According to results of barium chloride test, the second hypothesis that the urine of pregnant jennies mixed together with 1% solution of $BaCl_2$ will not coagulate was rejected.

5.4 Seed germination test

Altogether, 10,800 seed were included in the experiment, 50 % of seeds were mung beans and 50 % wheat seeds. 3,600 seed were used for testing non-pregnant as a control, 7,200 seed were used for testing samples from pregnant females.

5.4.1 Evaluation of seed germination

Firstly, the evaluation was done according to pregnancy status. The seed germination was tested according to seed type, urine concentration, and day of experiment, see Table 8. The only significant result was in wheat 1:14 in Day 3 when the number of germinated seeds was higher in non-pregnant females (unpaired t-test: $t = -3.52$; $df = 17$; $p < 0.01$), see Figure 12.

Table 8 The results of seed germination test (Kruskal-Wallis test), significant results are marked by bold text

Seed type	Urine concentration	Day of experiment	Result of K.-W. test (H)	P value
Wheat	1:4	1	0.11	0.74
		2	0.37	0.54
		3	0.46	0.50
	1:14	1	0.40	0.55
		2	2.05	0.15
		3	**5.27**	**0.02**
Mungo	1:4	1	1.62	0.20
		2	0.62	0.43
		3	0.33	0.56
	1:14	1	0.03	0.86
		2	0.01	0.93
		3	0.13	0.72

In non-pregnant jennies, the influence of season on seed germination was found in Day 1 (Kruskal-Wallis test: H (3, N = 72) = 10.72, p = 0.01 and in Day 2 (Kruskal-Wallis test: H (3, N =

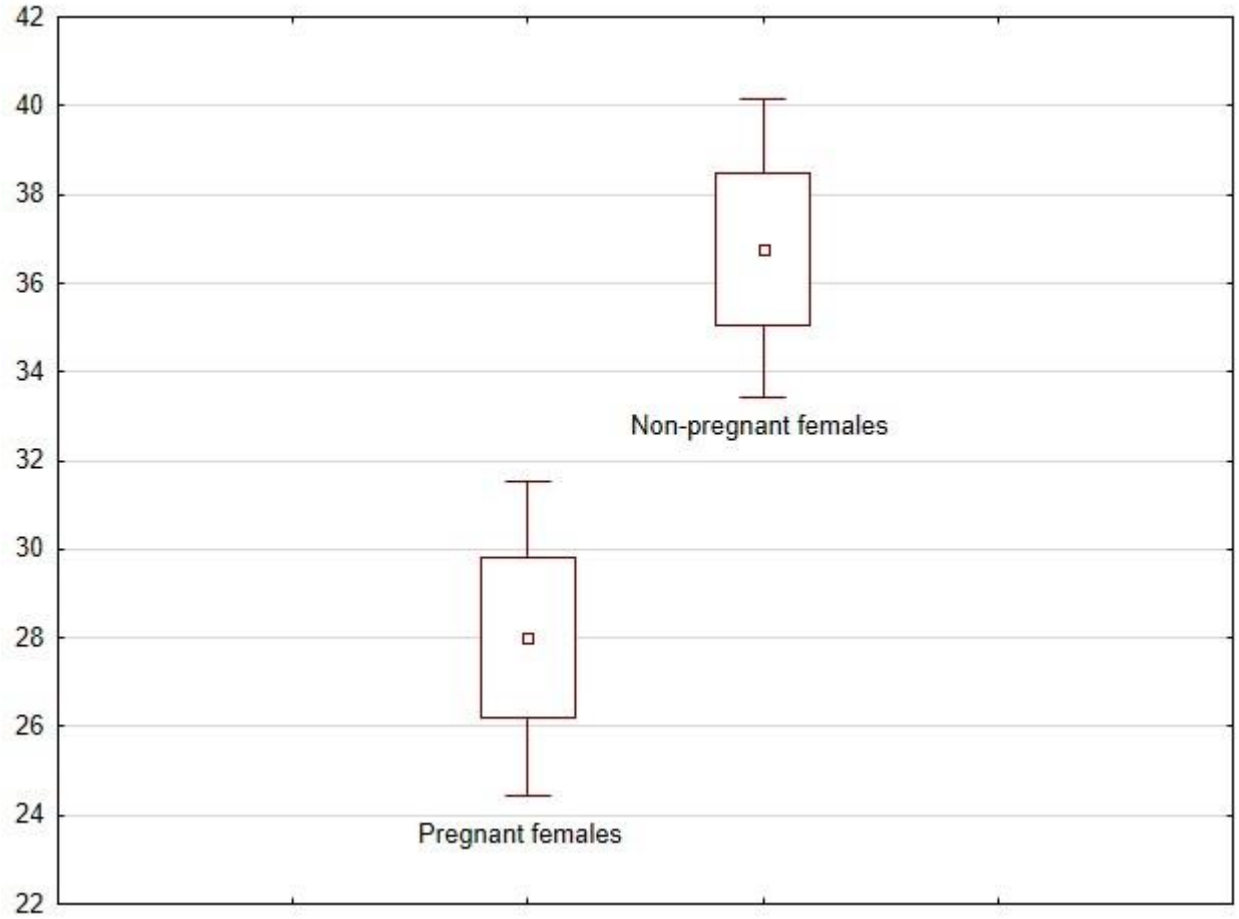


Figure 12: Difference in number of germinated seeds in trial with wheat dilution 1:14 between and pregnant and non-pregnant females (y axis represents number of germinated seeds)

72) = 7.62, p = 0.05). No statistically significant influence was found in Day 3 (Kruskal-Wallis test: H (3, N = 72) = 5.02, p = 0.17).

In pregnant jennies, number of germinated seeds were significantly affected by the season. In Day 1 (Kruskal-Wallis test: H (3, N = 144) = 8.11, p = 0.04), in Day 2 (Kruskal-Wallis test: H (3, N = 144) = 11.42, p < 0.001), and in Day 3 (Kruskal-Wallis test: H (3, N = 144) = 9.18, p = 0.03).

5.4.2 Evaluation of seeds shoot length

Generally, there was found no statistical difference between shoot length of seeds (both mung and wheat seeds) and pregnancy status (Kruskal-Wallis test: H (1, N = 216) = 1.82, p = 0.18), neither in thirds of pregnancy (Kruskal-Wallis test: H (3, N = 216) = 2.68, p = 0.44), nor in halves of pregnancy (Kruskal-Wallis test: H (3, N = 216) = 2.33, p = 0.31). The concentration and type of seed significantly influenced the shoot length (Kruskal-Wallis test: H (3, N = 216) =

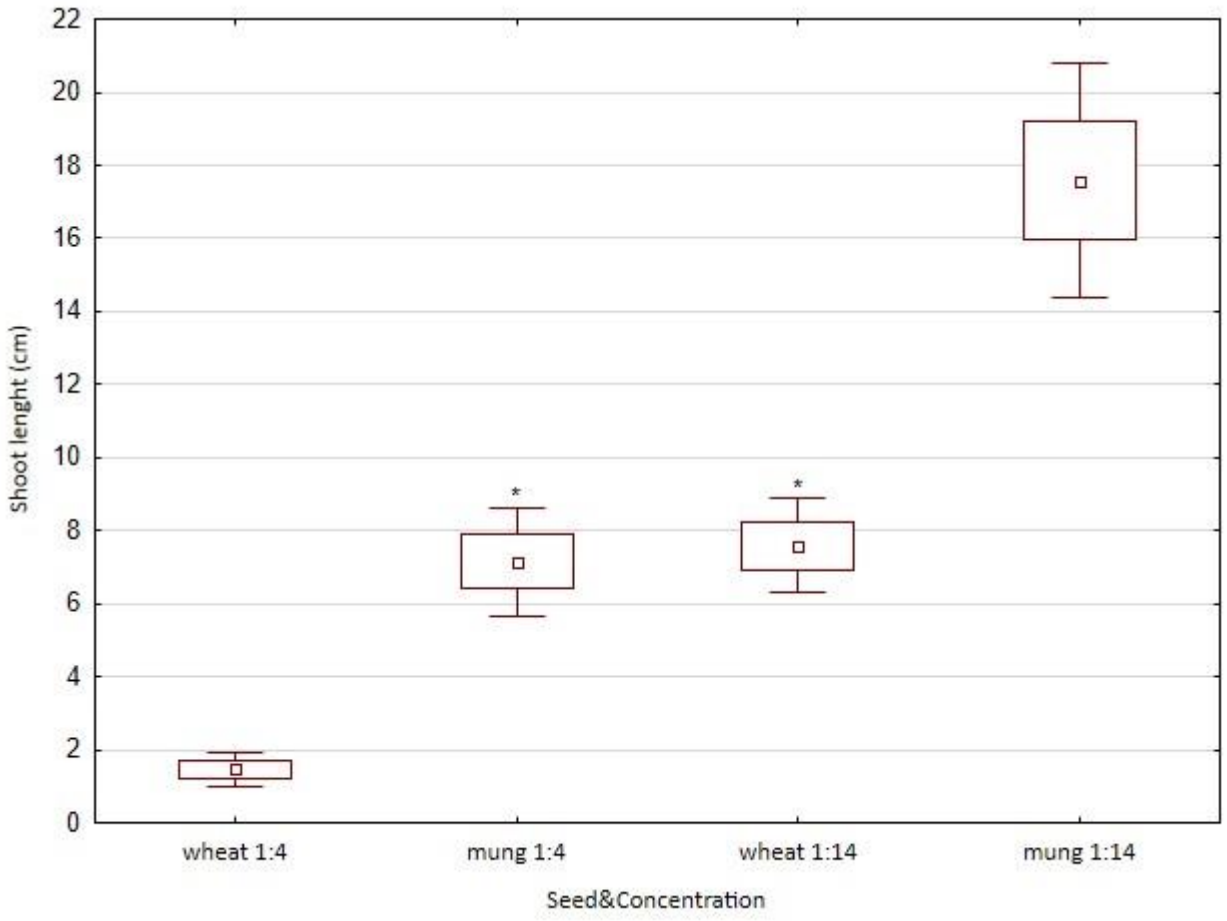


Figure 13: Difference in shoot length according to type of seed and concentration.

*** An asterisk marks groups which have no statistically significant difference to each other, other treatments (pairs) showed significant differences**

112.09, p < 0.001). To be more specific, statistically significant difference between all types of seeds and it´s concentrations with exception of wheat 1:14 and mung 1:4 where no significant differences were found (Multiple comparison test, p = 1.00), see Figure 13.

Seeds shoot length were evaluated according to seed type, urine concentration, and general pregnancy status. The significant relationship between shoot length and pregnancy status was highlighted, see Table 9.

Table 9 **The evaluation of seed shoot length according to pregnancy status (Kruskal-Wallis test), significant results are marked by bold text**

Seed type	Urine concentration	Result of K.-W. test (H)	P value
Wheat	1:4	0.27	0.61
	1:14	**6.36**	**0.01**
Mungo	1:4	0.04	0.83
	1:14	0.34	0.55

The significant results of wheat seed in dilution 1:14 showed longer shoot length in non-

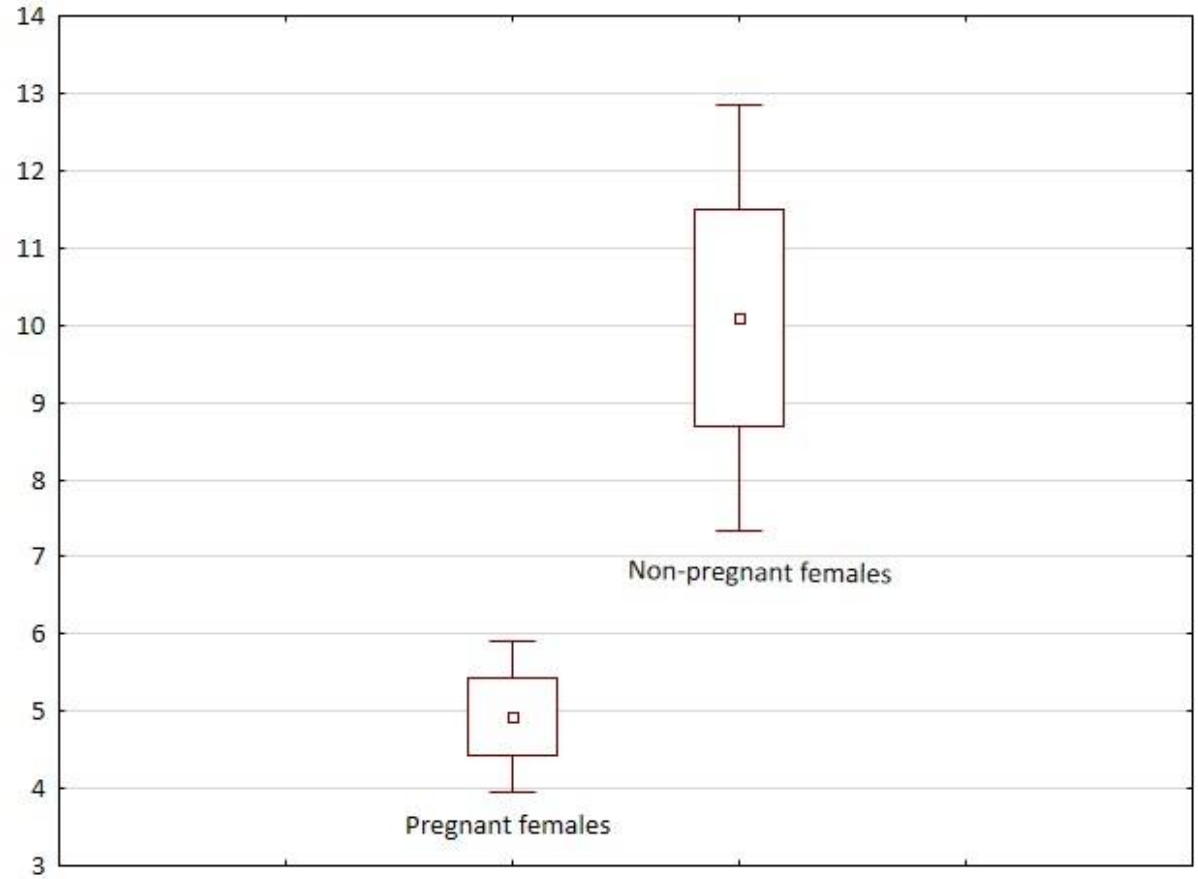


Figure 14: Difference in shoot length in trial with wheat dilution 1:14 between and pregnant and non-pregnant females (y axis represents seed shoot length in cm)

pregnant females compared to pregnant females (unpaired t-test: t = -3.77; df = 17; p < 0.01), see Figure 14.

The results of shoot length were evaluated according detailed status – third of pregnancy. The significant result was highlighted, see Table 10.

Table 10 **The evaluation of seed shoot length according to third of pregnancy (Kruskal-Wallis test), significant results = bold text**

Seed type	Urine concentration	Result of K.-W. test (H)	P value
Wheat	1:4	3.15	0.37
	1:14	**8.61**	**0.03**
Mungo	1:4	1.78	0.62
	1:14	0.81	0.85

Significant relationship was also searched between shoot length and half of pregnancy. Results are displayed in Table 11.

Table 11 **The evaluation of seed shoot length according to half of pregnancy (Kruskal-Wallis test), significant results = bold text**

Seed type	Urine concentration	Result of K.-W. test (H)	P value
Wheat	1:4	2.61	0.27
	1:14	**6.61**	**0.04**
Mungo	1:4	0.05	0.97
	1:14	0.35	0.84

There was found no statistically significant relationship between shoot length and season of sampling in pregnant jennies (Kruskal-Wallis test: H (3, N = 144) = 8.47, p = 0.04). Also no relationship found in non-pregnant jennies (Kruskal-Wallis test: H (3, N = 72) = 4.26, p = 0.23).

6. Discussion

The possibility to take obtain urine samples noninvasively may be considered as real. Since it is sometimes time-consuming to obtain the sample, it would be useful to establish quick method of sample colleting. For instance, it would be useful to set up a manual of urination trigger. If the donkeys are stabled, I would recommend to keep the jenny on rubber floor with slope so the urine would drain into groove and then can be easily collected. However, such stable can be just an experiment since the building costs would be higher than veterinary ultrasound examination. Nevertheless, since it is easy to obtain faces compared to urine, future research might consider more testing on feces.

6.1 Urinalysis

pH values measured by Duotest® double zone pH-indicator papers were corresponding to standard pH range in equids according to Harkins et al. (1996), Stämpfli and Carlson (2001), Robert et al. (2010) and Goren et. al (2014). Result from DekaPhan® Leuco test strips also responded to standard pH range. However, according to ErbaGroup (2015), the results from DekaPhan® Leuco test strips may be influenced by foreign substances to acidic and alkaline based. The sample collection was done outside on pastures, however the urine was collected into plastic cup so contamination of the samples was minimized.

Because no information about density and specific gravity in donkeys was found, the results were compared with horses' values. Density was slightly higher than regular in horses. However, the mean density was similar to Schumacher and Moll (2011). Results of specific gravity counted from density in donkeys were in accordance with research of Schumacher and Moll (2011), Adam (2012), and Schott (2012). However, specific gravity measured by DekaPhan® Leuco test strips did not correspond to results of authors above. Nevertheless, results from DekaPhan® Leuco test strips are considered as less exact since these strips were primary created for human urinary analysis. According to ErbaGroup (2015), the results from DekaPhan® Leuco test strips might be influence by pH higher than 6.5 so all specific gravity values were potential influenced by high pH. Another factors influencing the specific values might be connected to nutrition of donkeys since the specific gravity significantly varied among the farms. Describing these influencing factors can be an aims for future research.

Leucocytes, nitrites, glucose, ketones, and hemoglobin did not occur in any sample as it is correct in heathy equids according to Parrah et al. (2013). Blood, which showed in one sample, indicates extremely high specific gravity according to ErbaGroup (2015). However, this theory does not suit to the female Rainy with the light positive blood result since her spec. gravity was in the lowest measured level. Generally, according to Parrah et al. (2013), blood in urine may indicate disorders such as: kidney damage, infection, kidney or bladder stones, and kidney or bladder cancer or blood disorders. Proteins are supposed to be negative in heathy equids according to Parrah et al. (2013). 61.11 % of positive protein samples in donkeys can be caused by alkaline urine (pH > 8) or high buffering capacity of the urine (ErbaGroup, 2015). Others indicators are for example following: renal disease, fever, congestive heart failure (CHF), hypertension or tumors. However, almost 91 % of positive samples had the lowest measurable level of proteins. Urobilinogen was another parameter which was higher than is expected according to Parrah et al. (2013). Reason for this not standard urobilinogen levels could light which is one of the influencing factors according to ErbaGroup (2015). Higher levels of bilirubin could be influenced by high concentration of urobilinogen and light (ErbaGroup, 2015).

6.2 Cuboni reaction

This test was expected to be the most suitable for donkeys since it is finely working in horses, as it was proved by Cuboni (1934) and Purohit (2010). The hormone levels are mostly similar during pregnancy in horses and asses so the results of Cuboni reaction lived up to my expectations.

Results obtained from SVI were positive or negative so there was no dubious reaction like in camels tested by Fedorova et al. (2013; in press). In donkeys, the potential of usage is better than in camels. Camels showed same success in testing the 3rd third of pregnancy (100 %), however, donkeys reached 100 % success also in the whole 2nd half of pregnancy. On the other hand, the results from cows of domestic cattle and alpacas according to Skálová et al. (2014) were not satisfactory at all.

Nowadays, Cuboni reaction was not applied for testing donkey´s pregnancy but as showed in this study, it has a great potential. Not only that the results from 2nd half of pregnancy are correct, the Cuboni reaction was not affected by the season. Czech donkey breeders usually use ultrasound for pregnancy testing. According to Purdy (2005c), the application of ultrasound is possible in the end of first months of pregnancy. Cuboni reaction is

certainly not applicable in such early stage of pregnancy, however most of Czech breeders have donkeys as pet animals. Therefore, the early pregnancy diagnosis in not so indispensable.

6.3 Barium chloride test

Donkeys did not reach such promising results as cows of domestic cattle (Maslov and Smirnov, 1965) or pigs. Barium chloride was described as not suitable for pregnancy testing in camels (Fedorova et al., in press) and in alpacas (Skálová et al., 2014). Unfortunately, donkeys can be also classified as unsuitable animal species. Donkeys showed large percentage of false-negative results. However, season is not the reason of false-negative results since it was found not effect on the results. In field conditions, it is not clear what factors may affect the results. The big issue might contamination by foreign substances which may cause acidic and alkaline changes in the urine.

6.4 Seed germination test

In donkeys, most of the trials showed no relations between pregnancy status and seed germination test results. This results did not correspond to results of Dilrukshi and Perera (2009a) and Krishna and Veena (2009). In alpacas (Kubátová et al., 2014) seeds grew and geminated better in urine of pregnant females but this situation did not occurred in donkeys. As in sheep (Islam, 2013), it was expected to find significant difference between pregnant and non-pregnant groups in germination percentage and shoot length of germinated wheat. However, in donkeys only wheat seed with urine concentration 1:14 on Day 3 lived up to expectations. This result do not correspond to results from alpacas (Kubátová et al., 2014) where the significance was found in mung seeds with urine concentration 1:4. Nevertheless, urine concentration 1:4 seems more demonstrative for pregnancy testing.

In pregnant jennies seed germination was effected by season no matter which seed and urine concentration was used. Non-pregnant jennies samples showed influence of season in results from Day 1 and Day 2. Results from Day 3 were not influenced by the season. However, pregnant jennies samples were influenced by the season during whole trial. Season influence was observed also in alpacas (Kubátová et al., 2014). It is an important factor which can influence the results of seed germination test.

Shoot length were not reliably influenced by the pregnancy status of donkeys, such it was in cows of domestic cattle (Veena and Narendranath, 1993; Dilrukshi and Perera, 2009b;

Krishna and Veena, 2009; Narayana Swamy et al., 2010; Perumal, 2014; Rine et al., 2014) and in sheep (Islam, 2013). However, there was exception in wheat seeds dilution 1:14 when the relationship between shoot length and pregnancy status was observed. So in non-pregnant jennies, the shoots were significantly longer compared to pregnant jennies shoots. This result do not correspond to results from alpacas (Kubátová et al., 2014) where the significance was found in mung seeds with urine concentration 1:4.

Season did not influence shoot length neither in pregnant, nor in non-pregnant jennies. This outcome is against results from alpacas studied by Kubátová et al. (2014).

7. Conclusion

Experiment showed that it is possible to collect urine samples non-invasively in donkeys. Because of urinalysis, the values for average pH and specific gravity of the urine were set up. This outcome may be useful for donkeys' breeders in Czech Republic.

The Cuboni reaction has a great potential in pregnancy diagnosis in donkeys. 100 % correct results in second half of pregnancy are very promising. Season did not influence the results so it might be useable also in tropical climate. As it was written in methodology, Czech breeders keep donkeys mostly as a pet animals in small herds so for future research, country with larger donkey population would be more suitable. I would recommend more detailed research in future. It would be interesting to determine the exact months of pregnancy, in which can be the Cuboni reaction successfully applied.

On the other hand, Barium chloride test appeared to be absolutely unsuitable for donkeys. That is pity since it is very quick test and it is not influenced by the season. However, the sample collection was provided on the pasture so spoilage by some foreign particles is not inconceivable.

The seed germination test showed fairly unconfident results since the pregnancy status impacted only wheat seeds in dilution 1:14. But it is very important to highlight the influence of the season. Therefore the further research should be provided during one season. The future research could also focus on shortening and simplification of the procedure.

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