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Pregnancy Diagnosis from Urine in Even-toed Ungulates

Master Thesis

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Authorship Declaration:

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Abstract

Pregnancy Diagnosis from Urine in Even-toed Ungulates

This master thesis reviewed possible non-invasive pregnancy diagnostic methods in ungulates with special attention to diagnosis from urine. The practical part was focused on pregnancy diagnosis from urine in alpacas (*Vicugna pacos*). The aims of the thesis were to examine the possibility of catching fresh urine directly from female alpacas and to evaluate three pregnancy diagnostic tests from urine in alpacas – the seed germination test, the barium chloride test, and the Cuboni reaction. The research was carried out in the period from April 2013 to February 2014 on the three Czech private farms. Urine was collected non-invasively into plastic cups fastened on a rod in 6-8 week intervals. In total, 60 urine samples were collected from twelve alpacas. Urinalysis was done in each sample. For the seed germination test, mung beans (*Vigna radiata*) and winter wheat seeds (*Triticum aestivum*) were used and the urine was diluted to 1:4 and 1:14 ratios. Germination rates were counted 24, 48, 72, 96 and 120 hours after establishment of experiments. Lengths of shoots were measured 120 hours after establishment of experiments. The barium chloride test was done with 5 ml of urine mixed together with 5 ml of 1% solution of barium chloride in the laboratory of the FTA CULS Prague. The Cuboni reactions were performed by the State Veterinary Institute Prague. In case of the seed germination test, it was found out that the urine of alpacas inhibited the germination and growth of seeds in general. Usually, seeds germinated and grew better in the urine of pregnant females than in the urine of non-pregnant females. Further research was recommended. No relationship was found between the real reproductive status of alpacas and the results of the barium chloride test and the Cuboni reaction, even if the accuracy was assessed for non-pregnancy versus the whole period of pregnancy, halves of pregnancy or thirds of pregnancy. It was concluded that the barium chloride test and the Cuboni reaction are not suitable for pregnancy diagnosis in alpacas.

Keywords: Cuboni reaction, barium chloride test, seed germination test, alpaca, gestation

Abstrakt

Diagnostika březosti z moči u sudokopytníků

Diplomová práce shrnuje informace o možných metodách neinvazivní diagnostiky březosti u kopytníků se zvláštním důrazem na diagnostiku z moči. Praktická část práce byla zaměřena na diagnostiku březosti z moči u alpak (*Vicugna pacos*). Cílem práce bylo ověřit možnost odběru čerstvé moči přímo od samic alpak a zhodnotit na ní tři metody diagnostiky březosti z moči – test klíčivosti semen, test chloridem barnatým a Cuboniho reakci. Výzkum probíhal od dubna 2013 do února 2014 na třech soukromých farmách v České republice. Moč byla sbírána neinvazivním způsobem do plastových kelímků připevněných na tyči v intervalu 6-8 týdnů. Celkem bylo získáno 60 vzorků moči od dvanácti alpak. U každého vzorku byl proveden rozbor moči. Pro test klíčivosti semen byly použity fazole mungo (*Vigna radiata*) a semena pšenice ozimé (*Triticum aestivum*) a moč byla ředěna v poměru 1:4 a 1:14. Počty vyklíčených semen byly počítány 24, 48, 72, 96 a 120 hodin od založení pokusů. Délky klíčků byly měřeny po 120 hodinách od založení pokusů. Test chloridem barnatým byl prováděn smícháním 5 ml moči s 5 ml 1% roztoku chloridu barnatého v laboratořích FTZ ČZU Praze. Cuboniho reakce byly vykonávány ve Státním veterinárním ústavu v Praze. V případě testu klíčivosti semen bylo zjištěno, že moč alpak inhibovala klíčivost a růst semen. Většinou semena klíčila a rostla lépe v moči březích samic v porovnání s močí nebřezích samic. U tohoto testu byl doporučen další výzkum. Nebyl nalezen žádný vztah mezi reprodukčním stavem alpak a výsledky testu chloridem barnatým a Cuboniho reakcí, a to ani v případě, že jeho přesnost byla testována nejen pro nebřezí a březí samice, ale i pro jednotlivé poloviny a třetiny březosti. Z toho lze vyvodit, že test chloridem barnatým ani Cuboniho reakce nejsou vhodné pro diagnostiku březosti u alpak.

Klíčová slova: Cuboniho reakce, test chloridem barnatým, test klíčivosti semen, alpaka, březost

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List of Abbreviations

ABA	abscisic acid
AI	artificial insemination
$BaCl_2$	barium chloride
CL	*corpus luteum*
CULS	Czech University of Life Sciences Prague
CVRL	crown vertebral rump length
E1S	oestrone sulphate
eCG	equine chorionic gonadotropin
EIA	enzyme immunoassay
FSH	follicle-stimulating hormone
FTA	Faculty of Tropical AgriSciences
H	hypothesis
H_2SO_4	sulphuric acid
HCl	hydrochloric acid
LH	luteinizing hormone
P4	progesterone
PdG	pregnanediol-3-glucuronide
RIA	radioimmunoassay
SVI	State Veterinary Institute
zoo(s)	zoological garden(s)

1. Introduction

The research methods in animals can be divided into two groups – invasive and non-invasive (Long et al., 2008). The differences and frontiers between these two groups are discussed frequently (Garshelis, 2006; Long et al., 2008). Generally, the invasive methods penetrate the skin of animals or invade the animal body through the orifices (Garshelis, 2006). In contrast, non-invasive methods enable to collect data without handling or capturing the animals. In some types of research, where differentiation of individual animals is not required, non-invasive methods provide data even without observing the animals. Collecting of urine belongs to non-invasive research methods (Waits and Paetkau, 2005), which are more and more popular nowadays (Garshelis, 2006; Kleiman et al., 2010).

Even-toed ungulates represent one of the most important groups of domestic animals. In the year 2011, five members of even-toed ungulates were placed on the world list of ten most bred domestic animals together with poultry, rabbits and hares (FAO, 2012), see Annexe 1. Alpacas (*Vicugna pacos*) and other camelids (lamoids and camels) were not included because their importance is local (Djemali and Alhadrami, 1998; Vilá Melo and Gutiérrez Vásquez, 2012). In spite of that, lamoids (also called New World camelids or South American camelids) with more than 8 million animals worldwide in 2011 (FAO, 2012) are also significant representatives of domestic animals.

The pregnancy diagnosis is an essential part of reproduction research. The analysis of papers on mammals in Zoo Biology journal can serve as a good example. In the period from 1982 to 1992, reproductive biology was the subject of 20.2% of studies and represents the third biggest area of research in this decade. Between 1996 and 2004, reproduction was the most important research area at all (Rees, 2011). In the analysis of 194 papers from different scientific journals from 2000 to 2011, reproduction research was on the second place after veterinary care and welfare area (Kubátová, 2012). The reproduction is always on top among research areas, because it is a key factor in successful breeding management of livestock (Gargiulo et al., 2012) and wildlife too (Monfort et al., 1993).

2. Literature Review

2.1 Pregnancy diagnosis

Pregnancy diagnosis is essential for better reproductive management in animals. In domestic animals reproductive efficiency represents an important criterion for the production (Dilrukshi and Perera, 2009; Balhara et al., 2013), which leads to profitable animal husbandry. Early pregnancy diagnostics enables to detect the females, which did not conceived and should be rebred, treated (Balhara et al., 2013) or weeded out from reproduction, and usually subsequently culled (Lalrintluanga and Dutta, 2009; Bah et al., 2010; Holendová and Čechová, 2010; Perumal, 2014) in the shortest interval possible to prevent the losses.

Pregnancy diagnosis is very important also in wildlife or zoo animals. It enables to gather the information about the internal state of animals (Bashaw et al., 2010), to recognize pseudo-pregnancy (Dehnhard et al., 2010; Willis et al., 2010) and prenatal mortality (Willard et al., 1998; Lamb and Fricke, 2005), or to estimate the date of birth and be prepared for it (Kleiman et al., 2010). It also allows to detect the number of embryos/foetuses, to recognize their sex (Lamb and Fricke, 2005) and monitor their development (Suguna et al., 2008). Early pregnancy diagnosis also enables to change the nutrition of the females according to the pregnancy status (Kleiman et al., 2010).

Pregnancy diagnostic methods can be sorted out by many ways. From the animal point of view, the division into two large groups is important – there are invasive and non-invasive methods of pregnancy diagnosis (Kumar et al., 2013). The general principle for the differentiation of invasive from non-invasive methods was explained in the chapter 1. Introduction. Purohit (2010) divides methods of pregnancy diagnosis according to different criteria either into two or three groups. In case of the division into two groups, there are direct and indirect methods. In case of the division into three groups, there are visual, clinical and laboratory methods (Purohit, 2010). Wani et al. (2003) classified the methods as physical, chemical, biochemical, immunological and biological (Krishna Rao and Veena, 2009; Rao Krishna and Veena, 2009). Seven classes of pregnancy tests are distinguished by Cowie (1948): clinical, hormonal, enzymatic, biochemical, physiological, immunological and physical.

The pregnancy diagnosis should be reliable enough, user-friendly, examinant-, animal-, and concept-friendly (Gargiulo et al., 2012). It should be also simple (Krishna Rao and Veena, 2009), economical and time undemanding (Lalrintluanga and Dutta, 2009). Ultrasound fulfils all these requirements. It can be a very fast method when it is done by an experienced sonographer (Lamb and Fricke, 2005) and can be even used as a non-invasive method (Kähn et al., 1993; Hunnam et al., 2009; Aziz and Lazim, 2012). Ultrasound is often used in developed countries (Ndu et al., 2000a; Ndu et al., 2000b), both in domestic animals (Kähn et al., 1993; Lamb and Fricke, 2005; Hunnam et al., 2009) and wildlife (McNay, 2006; Kleiman et al., 2010). However, for developing countries, the ultrasound may be too expensive to get or keep, and too complicated to use for smallholders who are often illiterate (Ndu et al., 2000a; Ndu et al., 2000b).

For developing countries, other methods of pregnancy diagnosis are needed. They should be (besides requirements for methods in developed countries) cheap, affordable (Ndu et al., 2000a; Ndu et al., 2000b), farmer-friendly, non-invasive (Dilrukshi and Perera, 2009), and requiring neither restraining of animals nor specialists for interpretation of the results (Rao Krishna and Veena, 2009). According to Narayana Swamy et al. (2010) the methods for developing countries should be mainly door step techniques which can be used by farmers directly on farms with the help of cheap materials and with no requirements of special skills.

2.2 Non-invasive pregnancy diagnosis in ungulates

Trans-abdominal (trans-cutaneous) ultrasonography (Kähn et al., 1993; Hunnam et al., 2009; Aziz and Lazim, 2012) and hormone analysis of faeces, urine, milk (Bamberg et al., 1991; Kumar et al., 2013) and partially saliva (Sathe, 2012; Volkery et al., 2012) are common non-invasive methods of pregnancy diagnosis used in ungulates.

Other possible methods for non-invasive pregnancy diagnosis in ungulates are different tests based on examination of previously mentioned body secretions (Krishna Rao and Veena, 2009; Skalova et al., 2013) and excretions (Krishna Rao and Veena, 2009), as well as examination of fur (Cowie, 1948). Radiography without immobilization, observation of females (Kleiman et al., 2010; Reece, 2013), abdominal palpation (Kleiman et al., 2010), external ballottement (Cowie, 1948; Reece, 2013) and historically auscultation of the foetal heart beat (Cowie, 1948) represent other non-invasive pregnancy

diagnostic methods. All these methods have many advantages and disadvantages of their own.

2.2.1 Non-invasive pregnancy diagnosis by ultrasound and radiography in ungulates

Some advantages of the ultrasound were already mentioned in the previous chapter. Ultrasound and radiography are able to provide results immediately and detect not only the pregnancy itself, but also to determine the number of foetuses (Kleiman et al., 2010). However, both these methods cannot be used in all ungulates. Dangerous species and untrained animals pose a problem and must be immobilized during these diagnostic procedures (Kleiman et al., 2010). By this, the method turns its character into invasive one. There are also problems with radiography in large animals because the penetration of X-rays is limited in them (Reece, 2013). Besides, radiography is not reliably applicable until the foetal skeleton becomes visible (Evans and Anderton, 1992; Reece, 2013), and the effect of ionising radiation is potentially harmful for developing foetus(es) (Evans and Anderton, 1992).

In domestic animals, trans-abdominal ultrasonography for pregnancy diagnosis was used in cattle (Hunnam et al., 2009), sheep (Kähn et al., 1993; Aziz and Lazim, 2012), goats (Hesselink and Taverne, 1994), pigs (Scharfe et al., 1997), horses (McGladdery, 1999), llamas (Haibel and Fung, 1991), and alpacas (Mialot and Villemain, 1994; Knight et al., 1995; Smith and Hollingshead, 1999; Volkery et al., 2012).

In llamas and alpacas, trans-abdominal ultrasonography was found to be accurate from the 50^{th}-60^{th} day of pregnancy in the left flank, and from the 90^{th} day in the right flank (Hoops and Kauffold, 2013). Sufficient accuracy in the left flank is achieved in shorter time period because the majority of all pregnancies in the New World camelids occur in the left uterine horn (Picha et al., 2013), see chapter 2.4.1 Reproduction in female alpacas.

Trans-abdominal ultrasonography is also used in wild ungulates (Walton et al., 2014). Nowadays, animals in zoos are trained for ultrasound examination (Kleiman et al., 2010). There are many reviews dealing with the ultrasound examination for pregnancy diagnosis in various animals, e.g. the review of Kähn (1992).

2.2.2 Non-invasive pregnancy diagnosis by observation in ungulates

During pregnancy, changes in behaviour (Alarcón et al., 1990) and appearance (Kleiman et al., 2010) of females can be observed. The first sign of pregnancy is usually non-returning into oestrous, but this sign is unreliable (Muhammad et al., 2000). Reasons of unreliability have been described, e.g. by Purohit (2010).

In later stages of pregnancy following changes may be observed: swollen and enlarged abdomen (Kleiman et al., 2010) followed by bellying down (Reece, 2013), increasing body weight (Khanvilkar et al., 2009), and udder enlargement (Smith and Baldwin, 1974). However, the mammary gland of alpacas does not increase in weight until 30 days to parturition (Bravo and Varela, 1993). Movements of foetus(es) were observed after drinking of cool water in cows (Arave and Walters, 1980) and excretion of dark yellow coloured urine was observed in pregnant camel females (Khanvilkar et al., 2009). However, the accuracy of observation as a pregnancy diagnostic method is usually low (Muhammad et al., 2000), and it should be considered as a supplementary diagnostic method to other diagnostic methods (Purohit, 2010).

Observing the reaction of a female on the presence of a male can be a very good method of pregnancy diagnosis in some ungulate species. This is a very helpful method in camelids. Reliability of observing if alpaca females show the oestrous behaviour in the presence of males were 84% and 88% at the 70^{th} and 125^{th} day of pregnancy, respectively. In llamas, it was 85% and 95% at the 75^{th} and 125^{th} day of pregnancy, respectively (Alarcón et al., 1990). According to Smith and Hollingshead (1999), the accuracy can be even more than 90%.

A similar diagnostic method exists in Arabian camels. Cocking of the tail in pregnant females may be observed in the presence of a male camel from approximately the 15^{th} day of pregnancy. The test is quite accurate between the 15^{th} and 20^{th} day of pregnancy (Quzy et al., 2013). According to Tibary and Anouassi (1997), the same behaviour was observed in females of Bactrian camels, but not with such intensity as in females of Arabian camels (Purohit, 2010).

2.2.3 Non-invasive pregnancy diagnosis by abdominal palpation in ungulates

In ungulates, abdominal palpation as pregnancy diagnostic method is used in small domestic ruminants – goats (Ahmed et al., 1998) and ewes (Pratt and Hopkins, 1975).

A special kind of palpation is the external ballottement. It can be performed by a fist or knee of the manipulator. The pressure is exercised to the lower part of the abdominal wall on the right part of the female body in an inward and upward direction. Then the pressure is released and the floating movement of the foetus in foetal fluids can be detected (Reece, 2013). According to Adams (2007), this method is also used by Peruvian herdsmen after the 8th month of pregnancy in alpacas.

2.2.4 Non-invasive pregnancy diagnosis from fur in ungulates

Kosjakov test was developed for pregnancy diagnosis by sulphur content of the hair in pregnant and non-pregnant females. The quantity of sulphur was measured by the time required for decolourization of the methylene blue solution by the alkaline solution of the hair. However, the test was fundamentally considered as questionable (Cowie, 1948).

2.2.5 Non-invasive pregnancy diagnosis from saliva, milk and faeces in ungulates

Analysing of faeces, urine, milk and saliva mostly requires multiple collections of samples and laboratory testing which is not always possible (Kleiman et al., 2010). Results of these analyses are available with some delay, even if some on-farm kit for pregnancy detection is used (Simersky et al., 2007). The milk can be obtained just from lactating animals (Volkery et al., 2012), therefore, tests based on milk analysis cannot be done in heifers or dry-off animals (Kumar et al., 2013). Collection of milk and saliva requires higher degree of collaboration of the animals so tests based on these secretions are common just in domestic ungulates, but some exceptions exist (Czekala and Callison, 1996; Haberová et al., 2012). Testing of urine and faeces is preferred in zoological gardens (Kleiman et al., 2010). However, hormone analysis of faeces seems to be the most frequent in ungulates (Stoops et al., 1999; Graham et al., 2001). Acording to Kumar (2013), faecal steroids are the most practicable choice for non-invasive monitoring of reproductive status in all animals, both domestic and wild.

Hormone analysis of saliva, milk, faeces and urine for pregnancy diagnosis can be done by enzyme immunoassay (EIA) (Moriyoshi et al., 1996; Smith and Hollingshead, 1999; Stoops et al., 1999; Berger et al., 2006; Habumuremyi et al., 2014), radioimmunoassay (RIA) (Ohtaki et al., 1997; Garrott et al., 1998; Skolimowska et al., 2004; Karir et al., 2006), or gas/liquid chromatography-mass spectrometry (Combalbert et

al., 2010; Regal et al., 2012). However, EIA is the preferred method especially because it does not require usage, storage, and disposal of radioactive materials (Graham et al., 2001).

2.2.5.a Non-invasive pregnancy diagnosis from saliva in ungulates

The collection of saliva is done directly from the mouth, but is commonly considered as a non-invasive method of collection (Skálová et al., 2012; Skalova et al., 2013). Simple plastic disposable catering supplies (Haberová et al., 2012) or special kits for collecting (Volkery et al., 2012) can be used for sampling. The collection was examined in both domesticated ungulates (Moriyoshi et al., 1996; Ohtaki et al., 1997; Volkery et al., 2010; Haberová et al., 2012; Sathe, 2012; Skálová et al., 2012; Volkery et al., 2012) and ungulates in zoos (Czekala and Callison, 1996; Haberová et al., 2012).

Attempts to detect pregnancy from saliva were based on hormone analysis of saliva (Czekala and Callison, 1996; Ohtaki et al., 1997; Volkery et al., 2010; Sathe, 2012; Volkery et al., 2012), or analysis of saliva crystallization (Skálová et al., 2012; Skalova et al., 2013). Various hormones in saliva were monitored for pregnancy diagnosis, for example progesterone (P4) (Sathe, 2012), pregnanediol-3-glucuronide (PdG) (Volkery et al., 2010), oestrone sulphate (E1S) (Ohtaki et al., 1997), or relaxin (Volkery et al., 2012). Preservation and storage of saliva for hormone analysis were described e.g. by Czekala and Callison (1996), Sathe (2012), or Volkery et al. (2012).

For early pregnancy diagnosis from saliva in sow, a bovine milk P4 qualitative test EIA kit was successfully used by Moriyoshi et al. (1996). The hormone analysis could be a useful tool for pregnancy diagnosis not only in pigs (Ohtaki et al., 1997), but also in black rhinoceroses (Czekala and Callison, 1996). In contrast, hormone analysis of saliva seemed unsuitable in alpacas according to Volkery et al. (2010; 2012). In mares, further investigation was recommended by Sathe (2012). The saliva crystallization was verified as a method for early pregnancy diagnosis in cattle (Skalova et al., 2013).

2.2.5.b Non-invasive pregnancy diagnosis from milk in ungulates

Pregnancy diagnostic tests based on hormone examination of milk are known mainly in dairy cattle (Faustini et al., 2007; Friggens et al., 2008). However, they were examined also in beef cattle (Rainio, 1987), zebu cattle (Adeyemo, 1989; Kaul and Prakash, 1994), buffaloes (Karir et al., 2006; Karen et al., 2007), mares (Laitinen et al., 1981), ewes (Shemesh et al., 1979; Heshmat and Taha, 1984), goats (Murray and

Newstead, 1988; González et al., 2001), alpacas (Volkery et al., 2010; Volkery et al., 2012), and camels (Abdel Rahim and El-Nazier, 1987).

Hormone assays of milk were usually focused on concentrations of P4 (Friggens et al., 2008; Garmo et al., 2008), but also E1S (Zdunczyk et al., 2002), or relaxin (Volkery et al., 2012). Pregnancy-associated glycoproteins (Gajewski et al., 2008; Friedrich and Holtz, 2010; LeBlanc, 2013; Gajewski et al., 2014), pregnancy-specific proteins (Han et al., 2012), and early pregnancy factor (Coombes et al., 2003) were tested too.

Commercial on-farm kits based on assays of various hormones in milk already exist for pregnancy diagnosis in ungulates (Murray and Newstead, 1988; Rajamahendran et al., 1990; Dionysius, 1991; Engeland et al., 1997; Simersky et al., 2007). Commercial on-farm P4 kit, which was designed for dairy cattle, was tested for pregnancy diagnosis in alpacas with moderate reliability. However, measuring of P4 in milk in the first month of pregnancy can be a good alternative to trans-abdominal ultrasonography, which is not reliable enough at this time of pregnancy (Volkery et al., 2010; Volkery et al., 2012).

Other pregnancy diagnostic tests from milk also exist. These are milk-copper sulphate test (Krishna Rao and Veena, 2009) and milk-alcohol test used in cows (Cowie, 1948). Copper sulphate test is based on mixing of milk with 3% copper sulphate solution. The milk of a pregnant cow should coagulate while the milk of a non-pregnant cow should form a homogeneous mixture (Krishna Rao and Veena, 2009). Alcohol coagulation test is based on mixing of the same volumes of milk with pure alcohol. The mixture is then shaken and left to stand for 15 minutes to 3 hours. The milk of pregnant cows should coagulate. In contrast, milk of a non-pregnant cow should not show any changes (Cowie, 1948).

2.2.5.c Non-invasive pregnancy diagnosis from faeces in ungulates

Collecting faeces is very simple because it requires just collection from the ground without any disturbance of the animal (Bamberg et al., 1991). Because of this, it is the easiest non-invasive method to apply in zoos (Graham et al., 2001).

Hormone analyses of faeces for pregnancy diagnosis are used in domestic animals (Celebi and Demirel, 2003; Isobe et al., 2005), including alpacas (Smith and Hollingshead, 1999), zoo animals (Chapeau et al., 1993; Berger et al., 2006), and free-ranging wildlife (Garrott et al., 1998; Stoops et al., 1999; Skolimowska et al., 2004).

Monitoring of hormone levels for pregnancy diagnosis usually requires higher number of samples collected over a longer period of time (Skolimowska et al., 2004). In wildlife, this is impossible. But methods for detecting pregnancy from just one sample were developed (Garrott et al., 1998; Stoops et al., 1999).

Unconjugated steriod hormone metabolites of sex steroids were measured usually for pregnancy diagnosis from faeces (Lasley and Kirkpatrick, 1991). Pregnancy can be diagnosed according to the levels of progestagens including P4 (Garrott et al., 1998; Smith and Hollingshead, 1999; Isobe et al., 2005), or oestrogens (Bamberg et al., 1991; Chapeau et al., 1993). Faeces for hormone assays are stored frozen (Isobe et al., 2005). Storing conditions and problems related to sample preservation and storing are reviewed e.g. by Palme (2005).

2.3 Non-invasive pregnancy diagnosis from urine in ungulates

For non-invasive pregnancy diagnosis from urine the collection (IDEXX, 2006) sometimes followed by preservation and storage of urine is essential (Ribeiro et al., 2013).

Non-invasive pregnancy diagnosis from urine in ungulates can be done by hormone analysis of urine or by other special tests, such as the seed germination test (Veena and Narendranath, 1993), or various chemical tests like the barium chloride test (Maslov and Smirnov, 1965), Cuboni reaction (Cuboni, 1934), Kober method (Kober, 1938) or Lunaas method (Lunaas, 1961).

There are also so-called biological tests, such as Aschheim-Zondek test, Friedman test, toad test and others. In these tests, the urine of a female, which is undergoing pregnancy diagnosis, is injected into a female of other species, for example mouse, rat, rabbit doe or toad. Detailed information is summarized e.g. by Cowie (1948). These tests are not invasive for the females undergoing the diagnostics, but they are invasive for the females of other species used for the diagnostic testing. Because of this fact, in general these tests cannot be considered as non-invasive.

2.3.1 Non-invasive collection of urine

In the history, various kinds of harnesses equipped with collection containers were used for non-invasive urine collection in ungulates. Among the first ones was a device for collection of urine in male horses which was described by Vander Noot et al. (1965). All-purpose equipment for collection of urine from male cattle, sheep, and swine was

developed by Paulson and Cottrell (1984). It consisted of a waterproof cloth and a bag with a fastener (Paulson and Cottrell, 1984). Another equipment was designed for cattle and bison by Deliberto and Urness (1995). It was constructed from a urinary drainage bag, a belt of burlap and a plastic tube (Deliberto and Urness, 1995). Another urine collecting apparatus was developed by Van den Berg (1996) for mares.

The purely non-invasive method of urine collection is the free catch. It is the excellent method for animals, but it may be very exhausting for the collector (IDEXX, 2006). The keystone of the method is represented by a dry, clean collection container that is placed into the stream of urine. Great advantage of this method is the possibility to influence the quality of the sample by catching just the midstream of urine with lower content of bacteria, mucus and cells from urethra (IDEXX, 2006; Bassert and McCurnin, 2013). The disadvantage of the free catch method is potential significant bacterial contamination from the environment (Bassert and McCurnin, 2013).

Recently, various methods of non-invasive urine collection by the free catch were used in different animals. Collecting to the half-litre plastic cups fastened on a rod was used in Bactrian camels and cattle (Haberová et al., 2011; Haberová et al., 2012). Very similar equipment was used by Bravo et al. (1991b), and suggested by Bassert and McCurnin (2013) for llamas and alpacas. It was recommended to keep some distance from these animals not to disturb the urination. It is obvious when alpacas and llamas (both male and female) are going to urinate, because they usually urinate on communal dung piles and occupy a squatting position before urination. The stream of urine is going in caudal direction and the urination usually takes 30-60 seconds in these animals (Bassert and McCurnin, 2013). According to Volkery et al. (2012) the urine sampling in alpacas is difficult, time consuming, and in some females impossible at all. However, training of females may improve the chances for success (Volkery et al., 2012). In contrast, the collection of urine is easy in llamas according to Bravo et al. (1991b).

Rine et al. (2014) used plastic containers held in hands to collect the urine in cows, and this was also recommended by Bassert and McCurnin (2013). The same procedure was done by Ramsay et al. (1994) in various kinds of equid species, tapirs and rhinoceroses. Fresh, sterile container held in hand was recommended by Duncanson (2010) for horses. This statement was agreed by Bassert and McCurnin (2013), who suggested this method also for ewes and does. The collection in ewes and does should be expected immediately after rising of animals from the lying position on the ground because they tend to urinate

just at this moment (Bassert and McCurnin, 2013). Collecting urine to polythene bags (Lalrintluanga and Dutta, 2009), collecting cups (Bassert and McCurnin, 2013), and catching the urine outflow from the sloped animal box floor into plastic bowls with a wire mesh for faeces separation (Ndu et al., 2000b) was examined in sows. The timing is extremely important because adult sows urinate only 2-3 times a day (Bassert and McCurnin, 2013). According to Van Metre and Angelos (1999), a cap from a syringe case fastened on a coat hanger was used in miniature pigs for urine collection (Kurien et al., 2004).

In majority of experiments, collection of samples is done during spontaneous urination (Bravo et al., 1991b; Ndu et al., 2000b; Yang et al., 2003; Lalrintluanga and Dutta, 2009; Rao Krishna and Veena, 2009; Rine et al., 2014). However, induced urination may be exercised. In ewes, the urination may be provoked by occlusion of nostrils for a few seconds, but this is stressful for the animals (Bassert and McCurnin, 2013). Induced urination was used in experiments of Rine et al. (2014) in cows. It was induced by "...continuous stroking of the skin just below the vulva" (Rine et al., 2014). A similar procedure is possible in sows (Bassert and McCurnin, 2013). Volkery et al. (2012) used unspecified form of stimulation of urination in alpacas, but it was often unsuccessful.

It is also possible to influence indirectly the need of animals to urinate by cleaning the bedding or in llamas and alpacas by leading the animal to the communal dung pile (Bassert and McCurnin, 2013). In experiments of Seren et al. (1983) and Choi et al. (1987) even cellulose sponge inserted into vagina was used for collecting of urine in sows. However, this already represents a more or less invasive method of urine collection. Urethral catheterization and cystocentesis are also methods for animal urine sample collection, but both are invasive (IDEXX, 2006; Bassert and McCurnin, 2013).

Other non-invasive methods of collection of urine samples may also be used. It is possible to aspirate the urine from the floor of the enclosure (Ramsay et al., 1994), or from the ground immediately after the urination of an animal (Kirkpatrick et al., 1991a). Another possibility is the extraction of urine directly from soil (Kirkpatrick et al., 1991a; Kirkpatrick et al., 1991b), or usage of snow soaked with urine for analysis (Kirkpatrick et al., 1990).

2.3.2 Urine preservation and storage

The urine for experiments in relation to pregnancy diagnosis is usually used in fresh state immediately after the sampling. With this procedure no special storing conditions of urine are needed (Ndu et al., 2000a; Ndu et al., 2000b; Krishna Rao and Veena, 2009; Lalrintluanga and Dutta, 2009). Sometimes the urine was evaluated later with some delay, but usually after no more than just a few hours. In the seed germination test, the interval between the sample collection and the start of the experiment was 4-6 hours in experiments of Rao Krishna and Veena (2009) and Rine et al. (2014). In the experiments of Narayana Swamy et al. (2010) and Perumal (2014), testing of samples started on the same day, but the time interval was not specified. Also in these experiments, no special preservation was used.

In case that there is no possibility to evaluate the samples immediately after the sampling or in proper time, preservatives and/or regulated storing conditions are used. The conditions and preservatives differ according to the procedure for which the urine is used (Miki and Sudo, 1998; Ribeiro et al., 2013). Preservatives can influence the result of the upcoming procedure (Hoppin et al., 2006; Thierauf et al., 2008), their application is time-consuming, can be awkward, and in some cases also risky if they are toxic (Miki and Sudo, 1998). The refrigeration is the most used urine preservation technique because it is effective, simple and very cheap. It can preserve physical, chemical, microbial and cellular characteristics of the sample (Ribeiro et al., 2013). Haberová et al. (2011) stored the urine for the barium chloride test and the Cuboni reaction in a refrigerator for up to 14 days.

Research focused on factors, which affect the quality of stored animal urine, is performed mainly in pets, dogs (Padilla et al., 1981; Albasan et al., 2003), and cats (Albasan et al., 2003). The factors studied most often are the storage time and temperature (Padilla et al., 1981; Albasan et al., 2003). Albasan et al. (2003) studied how these two factors influence pH, specific gravity and crystal formation in the urine. It was found out that the storage time and temperature had no significant effect on pH and specific gravity of urine. In contrast, increased number and size of crystals was caused by refrigeration, and the number of crystals was higher with the longer time of refrigeration. Because of that, it is better to analyse the urine within 60 minutes after the collection according to Albasan et al. (2003). Proper length of storage time is dependent on the procedure, which is required for the evaluation of samples, because it can influence the results of the procedure (Hoppin et al., 2006). For example, the analysis of urine abnormal constituents and sediment should

be done within two hours after collection according to recommendations (Ribeiro et al., 2013).

Although the majority of experiments focused on pregnancy diagnosis from urine declare, that the evaluation of samples was done immediately after the sampling or with fresh urine, it still means some hours, and the rest of experiments were done with urine stored for even a longer time. According to Bassert and McCurnin (2013), the animal urine samples degrade quickly and should be analysed within 20 minutes, or within 2 days if immediately refrigerated.

For pregnancy tests based on urine examination the most important question is, if the stored urine sample has the same concentration of hormones and/or other factors, which may have influence on the results of these tests, as the fresh urine sample. Due to the lack of information about the factors, that potentially have an influence on some of the urine pregnancy tests (see chapters 2.3.3 Seed germination test and 2.3.4 Barium chloride test), there is also lack of information about how the urine should be properly preserved for these tests.

The Cuboni reaction is based on the reaction of free urinary oestrogens with sulphuric acid (H_2SO_4) (England, 2008), therefore, the stability of these hormones in urine is important in this test. The majority of experiments focused on hormone stability were done with human urine. According to Fuhrman et al. (2010), changes of concentrations of oestrogen and oestrogenic metabolites in human urine samples were less than 1% after 24 hour storage at 4 °C. In the experiment of Kjeld et al. (1977) with human urine without preservatives, it was detected that the levels of free oestradiol and P4 were not changed at 4 °C for up to 5 days. Changes of hormone levels were detected after 3 days in some samples at 18 °C, and at 37 °C changes were detected in two-thirds of samples after 18 hours (Kjeld et al., 1977). Similar results were obtained by Kesner et al. (1995). The activity of hormones in samples stored for two weeks without preservatives at 4 °C, 25 °C and 37 °C, was dependent on the temperature. It was shown that the higher the temperature, the lower the activity of hormones (Kesner et al., 1995).

Freezing is often used as a preservation technique of animal urine for hormone analysis (Todd et al., 1999; Habumuremyi et al., 2014). Glycerol can be used as a preservative to improve sample stability and to prevent loss of hormones (Livesey et al., 1983; Saketos et al., 1994; Kesner et al., 1995). Freezing of urine samples without any

preservatives was also used in experiments focused on hormone analysis of alpacas' urine (Volkery et al., 2012).

Important factor, which affects the efficacy of this technique, is the temperature of freezing. It is because at some temperatures losses of hormones can be higher (Livesey et al., 1983). In the experiment of Kjeld et al. (1977), the levels of free oestradiol and P4 in human urine did not change at -20 °C for up to 5 days. But the experiment of Livesey et al. (1983) with human immunoactive follicle-stimulating hormone (FSH) and luteinizing hormone (LH) showed that immunoactive FSH continuously disappeared from untreated samples at -25 °C with the half-life of 30 days. Even more rapid losses were detected at -20 °C. In contrast, less rapid losses were detected at -55 °C and +4 °C. The losses of immunoreactive LH were slower than losses of immunoreactive FSH (Livesey et al., 1983). Similar results were obtained by Saketos et al. (1994), who observed loss of activity of human urinary gonadotropins after 4 weeks of storage at -20 °C. In untreated samples of Kesner et al. (1995) stored at -80 °C for 24 weeks, changes in activity of human urinary LH, FSH and other reproductive hormones were found too. In contrast, in human urine samples stored at -80 °C by Fuhrman et al. (2010) for one year without any preservation, changes of concentrations of oestrogen and oestrogenic metabolites were less than 1%. For steroid evaluation and pregnancy diagnosis in animals urine samples of equid species, tapirs and rhinoceroses were stored at -4 °C for time periods lasting from less than 1 year to up to 10 years (Ramsay et al., 1994). During experiments in alpacas, urine for hormone analyses were stored at -20 °C (Volkery et al., 2012).

Another factor, which could change the quality of frozen urine samples, is the number of freeze-thaw cycles. However, it was observed by Fuhrman et al. (2010) that even 3 cycles had no consistent effect on human oestrogen and oestrogen metabolites concentrations. Urine samples of odd-toed ungulates for pregnancy diagnosis were tested even after several freeze-thaw cycles by Ramsay et al. (1994).

2.3.3 Seed germination test (Punyakoti test)

The predecessor of the seed germination test, which is also called Punyakoti test (Veena Ganesaiah, 2006), was probably one of the first pregnancy tests ever. It was described in the ancient Egyptian papyrus known as the Berlin Papyrus for the first time (Bayon, 1939). The information in the papyrus is probably 4 500 years old (Ghalioungui et al., 1963). The test was used in women and its principle was based on urinating on cereal

seeds. If the seeds grew, the woman was pregnant; if not, she was not pregnant (Bayon, 1939; Ghalioungui et al., 1963).

2.3.3.a Seed germination test in human

The potency of this method was tested by Hoffmann (1934) and later also by Ghalioungui et al. (1963), who confirmed that the urine of non-pregnant women always strongly or even completely inhibits the germination and growth of cereals. In human, the male urine also causes inhibition. Seeds in the urine of pregnant women usually grow, but not as a rule, because in this case the growth may also be inhibited. "It is concluded that when growth occurs, the urine is presumably that of a pregnant woman, but the reverse is not necessarily true" (Ghalioungui et al., 1963). In summary, the pregnancy can be only confirmed, but not excluded by this test.

The Berlin Papyrus also informs about the possibility to use two kinds of seeds and to detect the sex of the foetus by the kind of seeds that grows better. However, there is a problem with the names of cereals in the papyrus, because Egyptologists translated their names differently (Ghalioungui et al., 1963). According to Ghalioungui et al. (1963), the pairs of seed names were translated as wheat for boys and spelt for girls by Wreszinski (1909) and Dawson (1929), as wheat for boys and barley for girls by Iversen (1939), and as barley for boys and buckwheat for girls by Grapow et al. (1956).

The accuracy of the above-mentioned foetus sex test in human was assessed by Manger (1933). He considered faster growing of barley as an indicator for a girl, and faster growing of wheat as an indicator for a boy. The reliability in detecting the sex of the foetus was 80% in this way (Manger, 1933). Later, the test was marked as unreliable with just 47.5% reliability by Ghalioungui et al. (1963).

2.3.3.b Seed germination test in animals

The seed germination test was later examined in cattle by Veena and Narendranath (1993) in an attempt to find a new simple test for pregnancy diagnosis in cattle. They discovered that the germination and growth of wheat seeds were inhibited by urine of pregnant cows significantly more than by urine of non-pregnant cows. This pregnancy effect on seeds persisted even 2-3 months after parturition (Veena and Narendranath, 1993). The situation is completely opposite in human (Veena Ganesaiah, 2006). However, there is one thing, which is similar in the seed germination test in humans and cattle.

Majority of authors found out that the rates of germination and growth of seeds treated with urine (in both pregnant and non-pregnant, women and cows) were significantly lower than in seeds treated with distilled water (Ghalioungui et al., 1963; Veena and Narendranath, 1993; Dilrukshi and Perera, 2009; Krishna Rao and Veena, 2009; Rao Krishna and Veena, 2009; Narayana Swamy et al., 2010). However, the results of Rine et al. (2014) showed no significant differences in seed germination and shoot length between distilled water and urine of non-pregnant cows. Even no significant difference was found in seed germination and shoot length among distilled water and urine of non-pregnant and pregnant cows up to 21 days of pregnancy (Rine et al., 2014).

The results of Veena and Narendranath (1993), that urine of pregnant cows inhibits the germination and growth of seeds more in comparison with urine of non-pregnant cows, have also been confirmed by other authors (Dilrukshi and Perera, 2009; Krishna Rao and Veena, 2009; Rao Krishna and Veena, 2009; Narayana Swamy et al., 2010). However, significant inhibition of the germination and growth of seeds in urine of pregnant cows may be observed at least 28 days after conception (Rao Krishna and Veena, 2009; Rine et al., 2014).

Besides wheat seeds (Veena and Narendranath, 1993; Krishna Rao and Veena, 2009; Rao Krishna and Veena, 2009; Narayana Swamy et al., 2010; Rine et al., 2014), mung beans (Dilrukshi and Perera, 2009; Rao Krishna and Veena, 2009), sorghum, foxtail millet and paddy (Veena Ganesaiah, 2006) were also used for the seed germination test in cattle.

Cows are not the only animals in which the test has been examined. The seed germination test was extended also to buffaloes (Veena et al., 1997; Veena Ganesaiah, 2006; Dilrukshi and Perera, 2009; Rao Krishna and Veena, 2009), sheep, goats (Veena Ganesaiah, 2006; Rao Krishna and Veena, 2009), and mithuns (Perumal, 2014). In all these animals, the inhibition effect on seeds was higher with the urine of pregnant females than with the urine of non-pregnant females (Rao Krishna and Veena, 2009; Perumal, 2014).

2.3.3.c Reliability of the seed germination test

The reliability of the seed germination test in cows was detected as 100% by Krishna Rao and Veena (2009), who later specified, that the reliability was 68% in pregnant cows 28 days after artificial insemination (AI), and 100% in pregnant cows 35 and 45 days after AI (Rao Krishna and Veena, 2009). According to Rao Krishna and

Veena (2009), the earliest possible time point for pregnancy detection by the seed germination test in cows is 28 days after AI, but it is better to wait for a longer time to get the 100% accuracy, which is achieved somewhere between 28 and 35 days after AI. The same results were obtained by Rine et al. (2014).

It was concluded that the seed germination test is useful for pregnancy diagnosis also in mithun cows (Perumal, 2014).

2.3.3.d Differences in experimental design of the seed germination test

Some details of the seed germination test in cattle are different among scientists. These details are related to the degree of urine dilution with distilled water, amount of solution applied on seeds, different numbers of seeds in Petri dishes, soaking of seeds before the experiment, using of filter paper, time from inception of the experiment to counting of germinated seeds and seed shoot length measuring.

Veena and Narendranath (1993) used the ratio of urine per water 1:4 and applied 5 ml of this solution on 15 wheat seeds to each Petri dish, germination percentage was counted after 2 days and shoot growth was measured after 5 days. Dilrukshi and Perera (2009) used ratios 1:4, 1:10 and 1:14, applied 15 ml of diluted urine on 25 mung beans (which were soaked for half an hour in distilled water) to each Petri dish, germination percentage was counted after 1 and 2 days, and shoot growth was measured in 24 hour intervals up to 5 days.

Krishna Rao and Veena (2009) used the ratio 1:4, they did not specify the amount of applied solution or number of wheat seeds, and germination percentage was counted after 2 days. They did not measure the length of shoots. Instead of that, they observed the discoloration of the solution or seeds after 2 days as a sign of pregnancy (Krishna Rao and Veena, 2009). Later, they used the ratio 1:4 again; again, they did not specify the amount of solution applied on 8 wheat seeds and 8 mung beans in different Petri dishes, and germination percentage of both types of seeds was counted after 2 days as in the previous experiment. Newly they measured the shoot growth after 4 or 5 days in both types of seeds, and again they observed the discoloration of the solution or seeds after 2 days (Rao Krishna and Veena, 2009).

Narayana Swamy et al. (2010) used the ratio 1:4, and 15 ml of diluted urine was poured on 15 wheat seeds on filter paper in each Petri dish. They counted germinated seeds after 3 days and measured the shoot length after 5 days (Narayana Swamy et al., 2010).

The same procedure was also used in the study of Rine et al. (2014), the latest study about the seed germination test in cattle.

In mithuns, the ratio of urine per water 1:4 was used and 15 ml of the solution were applied on 15 paddy seeds on blotting paper in each sterile Petri dish. Germination percentage and shoot growth were measured after 3 days (Perumal, 2014).

Rao Krishna and Veena (2009) also tested if there is some difference between treating the seeds with distilled or tap water. It was found that there is no significant difference.

2.3.3.e Seed germination inhibition factor(s)

What causes the differences in germination between the seeds in urine of pregnant and non-pregnant animals has not been found out yet (Veena Ganesaiah, 2006). Following chemical substances or factors have been considered as culprits: pH (Veena and Narendranath, 1993), animal hormones (Nirmala et al., 2008), animal hormone metabolites (Rao Krishna and Veena, 2009), and plant hormones as auxins (Veena and Narendranath, 1993) or abscisic acid (ABA), which can cause dormancy of seeds (Veena Ganesaiah, 2006).

Veena and Narendranath (1993) rejected pH as a factor causing inhibition of seed germination and growth. They have proved that there are no significant differences in pH of urine of pregnant and non-pregnant cows. Oestrogen (oestradiol) and progesteron were rejected as inhibition factors by Nirmala et al. (2008) because they have proven that these hormones have no significant effect on seed germination and shoot length. Rao Krishna and Veena (2009) suggested that the factor is probably some metabolite, which is connected with changes of the reproductive tract that start with the conception and continue untill *post partum* period. They suspected some metabolite of P4 or oestradiol, or other pregnancy associated substance (Rao Krishna and Veena, 2009).

Higher concentration of ABA in urine of pregnant cows than in urine of non-pregnant cows was also discovered and because of this ABA could be one of the factors causing the inhibition of germination and shoot growth (Veena Ganesaiah, 2006). In contrast to this verdict there is an opinion of Rao Krishna and Veena (2009): "...plant growth regulators as auxins and abscisic acid are likely to be excreted in urine as and when the animals consume plants containing such substances." In summary, it is obvious, that

there is a lack of information about the exact nature of inhibition factor(s), and therefore, the research in this area should be a priority.

For the future, some attempts to develop a simple bioassay kit based on ABA reactions are planned. This kit should be readily used by farmers directly on farms in the rural areas to diagnose pregnancy in cows and buffaloes (Veena Ganesaiah, 2006). However, no such kit is available so far. The only possible choice are EIA kits (Biocompare, 2014), which are not affordable for common farmers in developing countries.

2.3.3.f Positives and negatives of the seed germination test

The seed germination test has many positives but also some negatives. The positives of the test are its simplicity, acceptable cost, and practicability in rural areas (Veena and Narendranath, 1993; Rine et al., 2014), non-invasiveness, welfare and no requirements of chemicals or sophisticated instruments (Veena Ganesaiah, 2006; Dilrukshi and Perera, 2009), possibility to do it directly at farms and by farmers themselves because of no need of special skills (Nirmala et al., 2008; Narayana Swamy et al., 2010), and high reliability of the test (Krishna Rao and Veena, 2009; Rao Krishna and Veena, 2009). The negatives are time demands (Krishna Rao and Veena, 2009) and relative laboriousness.

2.3.4 Barium chloride test

This method was developed by Maslov and Smirnov (1965) originally for cattle. It was discovered that addition of 1% solution of barium chloride ($BaCl_2$) to urine of non-pregnant cow causes a white precipitation. In contrast to that, addition of 1% $BaCl_2$ to the urine of pregnant cow does not cause any reaction. This method was tested in cattle also by Krishna Rao and Veena (2009).

Later, the barium chloride test was examined in pigs (Ndu et al., 2000a; Ndu et al., 2000b; Lalrintluanga and Dutta, 2009), Bactrian camels (Haberová et al., 2011), Arabian camels (Banerjee, 1974; Purohit, 2010), mares (Lalrintluanga and Dutta, 2009), ewes, and goat does (Ndu et al., 2000a; Ndu et al., 2000b).

2.3.4.a Reliability of the barium chloride test

According to Maslov and Smirnov (1965), the reliability of the test in cows is 95-100% from the 15th to the 210th day of pregnancy. However, this accuracy is obtained just

in cows kept in byres and it is not valid for cows on pasture. Maslov and Smirnov (1965) claimed that it is caused by higher urine oestrogen level, which is increased by oestrogens in the pasture. According to Krishna Rao and Veena, the efficacy of true positive reaction for pregnant and non-pregnant cows by the barium chloride test was 80% and 10% respectively, which means high degree of negative results for non-pregnancy. Because of that, the test was considered as not so useful in cows by them (Krishna Rao and Veena, 2009).

The effectivity of the barium chloride test in pigs was ascertained as 100% and 81% in non-pregnant and pregnant sows, respectively. In pregnant sows, the reliability increased with the duration of pregnancy from 55-59% in the first third of pregnancy (less than 38 days after conception) to 95-100% in later stages of pregnancy. To get at least 90-95% diagnostic accuracy, the test should be applied at least 39 days after AI or natural breeding (Ndu et al., 2000a; Ndu et al., 2000b). Later, the experiment in pigs was repeated by Lalrintluanga and Dutta (2009). The results corresponded to the results of Ndu et al. (2000a; 2000b). The accuracy of the test in pregnant sows was 64% in the 1st third of pregnancy (less than 38 days of pregnancy), 68% in the 2nd third of pregnancy (38 to 76 days of pregnancy), and 84% in the 3rd third of pregnancy (more than 76 days of pregnancy). In non-pregnant sows, the accuracy in different thirds of pregnancy was 84%, 88% and 88%, respectively. Obviously, the reliability of the test also increased in later stages of pregnancy (Lalrintluanga and Dutta, 2009).

During the experiments in pigs, it was also checked if the daytime of urine collecting and the parity of animals have some effect on the results of the test. Both of these factors finally had no effect on urine reaction (Ndu et al., 2000a; Ndu et al., 2000b).

The potency of the test in Bactrian camels was not confirmed. Haberová et al. (2011) "...did not find any clear relationship between the reproductive status of females and the results of barium chloride tests." According to Banerjee (1974), the reliability of the barium chloride test in Arabian camels was 85% between the 50th and the 90th day of pregnancy (Purohit, 2010).

2.3.4.b Differences in experimental design of the barium chloride test

The procedure of the test is different among scientist. One approach is mixing 5 ml of urine with 5 ml of 1% solution of $BaCl_2$ (Lalrintluanga and Dutta, 2009). Different amounts can be used too, but the ratio of urine and $BaCl_2$ solution should be maintained

(Krishna Rao and Veena, 2009). However, different approaches are also used. Ndu et al. (2000a; 2000b) added 5 drops of 1% $BaCl_2$ into approximately 2 ml of urine. Then the solution was shaken and left to stand for 5 minutes. After that, the result was evaluated. Haberová et al. (2011) used increasing amount of 1% solution of $BaCl_2$ (1, 5, 10 and 15 drops) that was added to the 5 ml of urine. Ohazurike (1990) used 2% solution of $BaCl_2$ in ewes and goat does (Ndu et al., 2000a; Ndu et al., 2000b).

2.3.4.c Precipitation preventing and inducing factor(s)

What causes the difference between reactions of the urine of pregnant and non-pregnant females has not been discovered yet (Ndu et al., 2000a; Ndu et al., 2000b; Lalrintluanga and Dutta, 2009). Maslov and Smirnov (1965) marked the high concentration of oestrogens and P4 in urine as a factor which prevents precipitation. According to Ndu et al. (2000a; 2000b) the differences could be caused by another factor different from oestrogens and P4. Their argument is based on the experiment of Ohazurike (1990), who tested the barium chloride test in non-pregnant ewes and goats in various phases of estrous cycle which means during oscillations of concentrations of oestrogen and P4 in blood. As a result there was precipitation of the solution in all cases (Ndu et al., 2000a; Ndu et al., 2000b). According to the opinion of Lalrintluanga and Dutta (2009), the factor is probably changing during pregnancy, and because of this the sensitivity of the test is increasing during the pregnancy in pregnant animals. P4 is suspected as the factor by these authors (Lalrintluanga and Dutta, 2009).

With the barium chloride test the situation is the same as with the seed germination test. There is lack of information about the exact factor(s), which cause(s) the different reaction of urine in pregnant and non-pregnant females, and therefore, the research in this area should be a priority.

2.3.4.d Positives and negatives of the barium chloride test

The positives of this test are its simplicity (Krishna Rao and Veena, 2009), low cost, availability to farmers, no need of special skills (Ndu et al., 2000a; Ndu et al., 2000b) and low time demands (Maslov and Smirnov, 1965; Lalrintluanga and Dutta, 2009). In contrast, $BaCl_2$ is a toxic substance, which is a significant negative (Ananda et al., 2013).

2.3.5 Cuboni reaction (Cuboni test)

The Cuboni reaction (sometimes also called Cuboni test) is named by professor Ettore Cuboni (1934), who developed it for pregnancy diagnosis in mares. The test is based on colour reaction of free oestrogens in urine with H_2SO_4 (England, 2008). The experiment in mares was repeated by Weber (1937), who tested also stallions and geldings.

In pregnant mares, positive results were obtained from the 132nd day of pregnancy. The urine of stallions reacted also positively. In contrast, the urine of non-pregnant mares and geldings reacted negatively (Weber, 1937). In other experiments it was found that negative reaction occurs also in infertile stallions (Cuboni, 1951). According to Hayes (2002), it is possible to detect the pregnancy of mares by the Cuboni reaction even earlier, circa 120 days after the conception. To get reliable results it is better to wait up to the 150th day of pregnancy, i.e. to the time of peak concentrations of placental oestrogens in plasma and urine, or even later, when the concentrations are still high, approximately up to the 300th day of pregnancy (England, 2008). At the end of pregnancy false negative reactions may occur in pregnant mares (Cuboni, 1951).

The reaction was tested also in cows (Weber, 1937; Dickenschied, 1939; Sardá and Sáinz-Sáinz-Pardo, 1940; O'Moore, 1947; Von Locvančič, 1969), pigs (Heller, 1940), ponies (Cox and Galina, 1970), Bactrian camels (Haberová et al., 2011; Fedorova et al., 2013), and Arabian camels (El-Ghannam et al., 1974).

Weber (1937) pointed out that the blood serum could not be used for the Cuboni reaction. The Cuboni reaction is applicable strictly to urine.

2.3.5.a Cuboni reaction in different kinds of animals

In pregnant cows positive results were obtained from the 8th month of pregnancy by Weber (1937) and from the 5th month of pregnancy by Sardá and Sáinz-Sáinz-Pardo (1940), but Weber (1937) pointed out that before the 8th month of pregnancy the results were not clear and unreliable. In general, the fluorescent reaction is weaker in cows than in horses (Von Locvančič, 1969). According to O'Moore (1947) the Cuboni reaction in cows is not reliable at all.

Positive and negative reactions from non-pregnant sows, and positive, negative and also dubious reactions from pregnant sows in different stages of pregnancy from the 1st to the 16th week were obtained by Heller (1940), who also tested the sows up to 21 weeks

after parturition and obtained all of the possible results too. On the basis of that, Heller (1940) declared that the test is not suitable for pregnancy diagnosis in pigs.

Modification of the Cuboni reaction was a keystone in the development of another unnamed chemical test for early pregnancy diagnosis in pigs. As the Cuboni reaction, the test was based on increasing excretion of oestrogens in urine of pregnant sows between the 3rd and the 5th week of pregnancy (and later again between the 11th week and parturition). As a part of the new test, the method for accurate distinguishing between doubtful and positive test results was developed. Positive tests in pregnant sows can be obtained in a short time period from the 21st to the 32nd day of pregnancy (Roth et al., 1941).

Positive reactions in pregnant pony mares were obtained beyond the 150th day of pregnancy by the modified Cuboni reaction (Cox and Galina, 1970).

In Bactrian camels, the accuracy of the Cuboni reaction was 100% in the 3rd third of pregnancy (Fedorova et al., 2013). Even 215 days before parturition positive reactions were noted in pregnant females (Haberová et al., 2011). False negative results in pregnant females occurred mainly in the 1st third of pregnancy and were associated with abortions. The latest false negative result was obtained 160 days before parturition (Fedorova et al., 2013). In non-pregnant females positive and dubious reactions occurred which may be caused by high oestrogen levels at the time of heat in camel females (Haberová et al., 2011), mainly during the winter when there is the breeding season of camels on the northern hemisphere (Fedorova et al., 2013).

The original Cuboni reaction in Arabian camels was examined by El-Ghannam et al. (1974). The results of the test were compared to the crown vertebral rump length (CVRL) as an indicator of the foetal development and progress of pregnancy. Dubious reactions were obtained from females carrying foetuses with CVRL of 26 cm. In case of foetuses with CVRL in the range of 26-55 cm, there was slightly positive fluorescence reaction of female urine. Sure positive reactions were obtained from females with foetuses that were reaching CVRL in the range of 60-120 cm (El-Ghannam et al., 1974).

2.3.5.b Differences in experimental design of the Cuboni reaction

The procedure of the Cuboni reaction was modified many times but always in order not to affect the results of the reaction negatively. The modifications of the reaction are described e.g. by Sardá and Sáinz-Sáinz-Pardo (1940), Roth et al. (1941), Jensen (1941, 1943), Boguth (1951), or Galina and Cox (1969). Many modifications are related to

solvents. Sardá and Sáinz-Sáinz-Pardo (1940) modified the test "...by extraction of the oestrogen by ether before the addition of benzene." Jensen used benzene as a solvent at first (1941), but later he successfully tried chloroform or ether instead of benzene with better results with ether (1943). The procedure, which is used by the State Veterinary Institute (SVI) Prague, is described in the chapter 4.4.3 Cuboni reaction.

There are also differences in the process of evaluation of results. The ultraviolet lamp is used according to Hayes (2002), but the SVI Prague uses just day light and dark background for the detection of the fluorescent reaction (Čadová, 2013).

2.3.5.c Positives and negatives of the Cuboni reaction

The great positive of this test in mares is its reliability. According to England (2008), the false reactions are very rare and the reaction is accurate. However, there are also different opinions. Duncanson (2010) declares that the reliability of the test is not higher than 80%, and that it is not used nowadays. This is in contrast to reality because the Cuboni reaction is performed in the SVI Prague.

Significant negative of the Cuboni reaction is toxicity of benzene, which is usually used as a solvent. It is a strong carcinogen. Also the use of acids can be dangerous (England, 2008). The SVI Prague applies the modification of the test and uses toluene instead of benzene. Toluene is toxic too, but less than benzene. The consequence of this modification is the less appreciable fluorescent reaction than the one caused by benzene (Čadová, 2013).

2.3.6 Kober method

Kober method was developed by Salomon Kober in 1931 for pregnancy diagnosis in mares. The method has been modified many times since its introduction. It is based on colorimetric evaluation of oestrone in urine. The procedure is based on mixing urine with concentrated H_2SO_4 or phenolsulfonic acid. The solution is then diluted with water and heated in order to develop the colour (Kober, 1938).

According to Cox and Galina (1970), the Kober method in mares was reliable from the 140^{th} day of pregnancy, but the procedure was complicated and time-consuming. Because of this, Kober method was declared as not suitable for routine usage.

2.3.7 Lunaas method

In 1962 Lunaas method was developed by Torleiv Lunaas for pregnancy diagnosis in pigs. The essence of this method is the modified Kober reaction. It is very simple and quick method based on detection of urinary oestrogens by colour reaction. Red colour and green fluorescence of the final extract are characteristic for the presence of oestrogens (Lunaas, 1961). The reliability of this test was found to be 90.3% in pigs (Cupps et al., 1966).

The method was also tested in mares (Lunaas, 1962). During the experiments of Cox and Galina (1970) there were problems to distinguish positive and negative results, therefore, this method was declared as unsatisfying for mares. Another author agreed with this statement – according to England (2008), the Lunaas method is more simple than the Kober method or the Cuboni reaction, but difficult to interpret. Cuboni reaction was recommended by Cox and Galina (1970) in contrast to Kober and Lunaas methods.

The Lunaas reaction was also tested in cows, but no positive reactions were detected in pregnant cows (Von Locvančič, 1969).

2.3.8 Hormone analyses of urine

Pregnancy diagnostic tests based on hormone examination of urine are known in domestic ungulates (Roser and Lofstedt, 1989; Yang et al., 2004), including alpacas (Volkery et al., 2010; Volkery et al., 2012), wild ungulates in captivity (Ramsay et al., 1987; Czekala et al., 1990; Monfort et al., 1993; Ramsay et al., 1994), free-ranging feral ungulates (Kirkpatrick et al., 1990; Kirkpatrick et al., 1991b), and wildlife (Kirkpatrick et al., 1991a; Kirkpatrick et al., 1992).

Conjugated steriod hormone metabolites of sex steroids were usually measured for pregnancy diagnosis in urine (Lasley and Kirkpatrick, 1991). Hormone assays of urine were usually focused on concentrations of oestrogen (Ramsay et al., 1987), oestrone conjugates (Monfort et al., 1993; Ramsay et al., 1994), including E1S (Czekala et al., 1990; Bravo et al., 1996; Volkery et al., 2012), or P4 (Volkery et al., 2010; Volkery et al., 2012), and its metabolite PdG (Kirkpatrick et al., 1991a; Ramsay et al., 1994; Yang et al., 2004). Equine chorionic gonadotropin (eCG) was also tested in some species (Roser and Lofstedt, 1989; Ramsay et al., 1994).

In alpacas, measuring of PdG in urine is suitable for early pregnancy diagnosis. However, it is recommended to confirm the pregnancy by trans-abdominal ultrasound later in its course (Volkery et al., 2010; Volkery et al., 2012).

Field dipstick tests for pregnancy diagnosis from urine in ungulates already exist (Ramsay et al., 1994). Commercial dipstick EIA, developed to detect eCG in blood, was also used to detect eCG in urine of mares (Roser and Lofstedt, 1989).

2.4 Reproduction and urinary hormones during pregnancy in alpacas

Reproduction of lamoids and processes connected with it have been more studied in domesticated species – llamas (*Lama glama*) and especially alpacas (*Vicugna pacos*), than in wild species – vicunas (*Vicugna vicugna*) and guanacos (*Lama guanicoe*) (Fernández-Baca, 1993; Vaughan and Tibary, 2006). However, all these species share common reproductive characteristics (Fernández-Baca, 1993). They are genetically similar, can interbreed, and have fertile offspring (Adams, 2007; Valenzuela-Estrada et al., 2012).

Alpacas together with llamas are important domesticated animals with significant economic impact (Vaughan and Tibary, 2006), especially in their native South America (Bradford et al., 1989). However, alpacas together with llamas have gained international popularity (Smith et al., 1994; Gauly, 1997), and their numbers have increased in Europe (Gauly and Bourke, 1997). Understanding of the reproduction as a complex event is important for the breeding management and for production as a whole (Vaughan and Tibary, 2006).

2.4.1 Reproduction in female alpacas

There are some special features of reproduction in alpacas and other lamoids in contrast to other domestic ungulates (Vaughan and Tibary, 2006; Knauf et al., 2008). Their sexual behaviour, copulation time and other reproduction aspects (Adams, 2007), like induced ovulation, absence of cyclic sexual activity, or relatively long pregnancy (Hoops and Kauffold, 2013), are extremely different from other domestic ungulates (Adams, 2007).

2.4.1.a Reproductive anatomy

Various parts of alpaca female reproductive tract are similar to other domestic animals. Ovaries are similar to cow´s ovaries but they are smaller (Adams, 2007). The

oviducts are long and squiggly. Alpacas have bicornuate uterus with the larger left horn (Vaughan and Tibary, 2006; Adams, 2007), where the majority of all pregnancies occur (Bravo and Varela, 1993; Fernández-Baca, 1993; Sumar, 1999; Hoops and Kauffold, 2013; Picha et al., 2013). However, this is not caused by a limited function of the right ovary, because ovaries are equally active (Fernández-Baca, 1993; Sumar, 1999). The vagina may be longer than 20 cm (Adams, 2007), and leads to the vulvar opening (Vaughan and Tibary, 2006).

2.4.1.b Reproductive physiology

Female alpacas reach puberty approximately at the age of 12 month (Fernández-Baca, 1993), or when they achieve 50% of the adult body weight (Sumar, 1999). For the first time they are bred at the age of 12-18 months (Adams, 2007).

In the Andean region female alpacas show reproductive seasonality (Sumar, 1999), which is probably caused by breeding management, when males and females are kept together (Fernández-Baca, 1993). In contrast, they act like non-seasonal breeders in conditions of Northern Hemisphere (Sumar, 1999), where males and females are usually kept separately (Fernández-Baca, 1993). Significant follicular activity is obvious throughout the whole year (Bravo and Sumar, 1989).

Adult female alpacas do not show a typical oestrous cycle. Instead, they have continuous oestrous with short periods of non-receptivity (Fernández-Baca, 1993). The ovulation is induced (Bravo et al., 1991b; Gauly and Bourke, 1997), which means that they ovulate just after copulation or hormone supply (San-Martin et al., 1968). The ovulation occurs within 12 to 24 hours (Gauly and Bourke, 1997), but according to different opinions even after 24-30 hours after copulation (Vaughan, 2011). San-Martin et al. (1968) suggest 26 hours. Multiple ovulations occur approximately in 10% of cases (Fernández-Baca, 1993), but alpacas usually have just one calf per gestation (Sumar, 1999).

When the ovulation is not induced in the right time, the non-pregnant ovulatory follicle regresses and is replaced by another growing follicle from the next follicular wave. This can be called alpacas' reproduction "cycle", which lasts 15 – 20 days (Adams, 2007). According to Sumar (1999), the time required for growth, maintenance and regression of a follicle is even shorter – around 12 days, and according to Vaughan (2011) it takes 10-22 days. In case of sterile mating, *corpus luteum* (CL) has a lifespan of about 13 days (Fernández-Baca, 1993). P4 concentrations in the blood are increased from the 5th day after

mating, they reach their maximum on the 7^{th}-8^{th} day, and decline on the 9^{th}-10^{th} day after copulation. After fertile mating, CL is formed and remains active throughout the pregnancy (Sumar, 1999).

2.4.1.c Copulation and mating behaviour

When an alpaca female is prepared for copulation, it is called to be "receptive". Receptive females usually occupy the prone (lying) position following some contact with a male (Adams, 2007), and the copulation takes place in this position (Fernández-Baca, 1993). Non-receptive females reject male´s attempts to copulate by spitting and running away. Males usually try to mate with all females, both receptive and non-receptive (Adams, 2007). The act of copulation is quite long – it can take up to 50 minutes (Fernández-Baca, 1993). Alpaca males strongly vocalize during mating. The frequency is also quite high – up to 18 copulations per day are possible (Adams, 2007).

In alpacas, repeat breeding syndrome (Tibary et al., 2001) and relatively high rates of embryonic and foetal mortality occur (Knight et al., 1995; Sumar, 1999; Brown, 2000), which leads to their lower reproductive efficiency (Fernández-Baca, 1993).

2.4.1.d Pregnancy and parturition

Non-invasive pregnancy diagnostic methods were described in previous chapters. Following invasive methods were used in alpacas: rectal palpation (Alarcón et al., 1990; Smith and Hollingshead, 1999), trans-rectal ultrasonography (Mialot and Villemain, 1994; Knight et al., 1995; Parraguez et al., 1997; Gazitua et al., 2001; Hoops and Kauffold, 2013; You et al., 2013), trans-vaginal ultrasonography (Catone et al., 2004), measuring of hormone levels in blood plasma (Volkery et al., 2010; Volkery et al., 2012), or serum (Bravo et al., 1996).

The length of pregnancy in alpacas may be considerably variable. It lasts according to San-Martin et al. (1968) 342-345 days, according to Fernández-Baca (1993) 342-350 days, according to Sumar (1999) 340-346 days, and according to Hoops and Kauffold 336-349 days (2013). The mean is 345 days according to Volkery et al. (2012).

The majority of parturitions happen in morning hours (Sumar et al., 1978). Alpaca females may show signs of oestrous 24 hours after parturition already (Fernández-Baca, 1993), but fertile mating is not accomplished until 15-20 days postpartum (Fernández-Baca, 1993; Sumar, 1999). However, it is still early after parturition. Therefore, alpaca

females can provide one calf per one year in spite of a long-lasting pregnancy (Sumar, 1999; Adams, 2007).

2.4.1.e Breeding management

In South America, the pasture breeding when male(s) and females are together on pastures as one herd is common. It the alternating (rotary) system of breeding there is just one male for a few females, and the males are regularly changed among female herds. Hand breeding, which is related to the intensive management, is not so common in this area (Adams, 2007).

Reproductive biotechnologies like AI, embryo transfer (Fernández-Baca, 1993; Vaughan, 2004), *in vitro* fertilization (Sumar, 1999), ovarian super-stimulation, nuclear transfer (Miragaya et al., 2006), follicular synchronization, oocyte maturation, and embryo cryopreservation have not been used extensively, but may be used in alpacas (Adams, 2007). These procedures would allow propagation of genetically superior animals, especially those with fibre of excellent quality (Miragaya et al., 2006).

Detailed information about reproduction in alpacas are reviewed e.g. by Smith et al. (1994), Gauly (1997), Brown (2000) or Vaughan and Tibary (2006).

2.4.2 Urinary hormone profile during pregnancy in female alpacas

In the urine of pregnant alpacas, changes of levels of E1S (Bravo et al., 1996; Adams, 2007; Volkery et al., 2012), P4, PdG (Volkery et al., 2010), or relaxin (Volkery et al., 2012) were measured for potential usage for pregnancy diagnosis.

Measurement of urinary P4 and relaxin concentrations was found unsuitable for pregnancy diagnosis in alpacas. There is overlap between concentrations of P4 in pregnant and non-pregnant animals without any significant cut-off values (Volkery et al., 2012). Further, concentrations of relaxin in the urine of pregnant alpacas are probably so low, that during experiments of Volkery et al. (2012), they were below the detection limit of EIA at any sampling time.

E1S concentration in urine peaks twice during pregnancy. The first peak is reached 21 days after mating (Bravo et al., 1996) and the second one is reached during the last month of gestation (Bravo et al., 1996; Volkery et al., 2012). According to Bravo et al. (1996), the first peak could indicate the early interaction between the mother and the

embryo and the second one could reflect foetal viability. However, the use of urinary E1S for pregnancy diagnosis is highly dependent on time of sampling (Bravo et al., 1996), and the possibility to diagnose pregnancy by the first peak of E1S was doubted by Volkery et al. (2012).

The only urinary hormone, which seems to be suitable for pregnancy diagnosis in alpacas, is PdG (Volkery et al., 2010; Volkery et al., 2012). Concentrations of PdG in urine have similar patterns as concentrations of P4 in plasma, which is probably the consequence of PdG urinary excretion as a metabolite of P4. The concentrations of PdG in urine are significantly higher in pregnant than in non-pregnant alpaca females. PdG concentration is increasing during the first month of pregnancy, with the peak at the end of the first month. The highest increase of concentration of PdG can be observed during the fifth month of gestation (Volkery et al., 2012).

The hormone profile during pregnancy in alpacas is very similar to llamas. Urinary hormone profile during pregnancy in llamas is described e.g. by Bravo et al. (1991a).

3. Aims of the Thesis

Aims of the thesis were:

1) to review the non-invasive methods of pregnancy diagnosis in ungulates, especially focusing on diagnosis from urine;
2) to examine the possibility of catching fresh urine directly from female alpacas (*Vicugna pacos*);
3) to verify by the research in practice the efficiency of three pregnancy diagnostic tests from urine in alpacas – a) the seed germination test, b) the barium chloride test and c) the Cuboni reaction.

Hypotheses of the thesis were:

H1: the urine of pregnant female alpacas will inhibit the seed germination and shoot length more than the urine of non-pregnant female alpacas;

H2: the urine of pregnant female alpacas mixed together with 1% solution of $BaCl_2$ will not coagulate;

H3: the urine of pregnant female alpacas will show green opalescence after the Cuboni reaction.

4. Material and Methods

The structure of the thesis was composed according to the Methodical Manual for the MSc Theses Writing of the Faculty of Tropical AgriSciences (FTA), Czech University of Life Sciences (CULS) Prague (FTA, 2014b). References were cited according to the Citation Rules of the FTA, CULS Prague (FTA, 2014a). Scientific names and taxonomy of lamoids were written according to Vaughan (2011).

4.1 Literature review

The literature review came out from the analysis of scientific publications (especially from the scientific database Web of Science) dealing with this topic. Articles were searched by following keywords: pregnancy, diagnosis, non-invasive, urine, Cuboni reaction, barium chloride test, seed germination test, and others.

4.2 Animals

The possibility of collecting fresh urine directly from female alpacas was tested on four alpaca females of huacaya type on the farm Centrum aktivního odpočinku – Jezdecká společnost Bitozeves o.s. of Mrs. Pavlína Kňavová in Bitozeves near Louny. After that, this farm was excluded from the research, because of lack of pregnancy diagnosis by the breeder, which could confirm the running pregnancy for the research purposes.

The urine for testing three pregnancy diagnostic methods was collected from twelve female alpacas of huacaya type from three Czech private farms. On each farm, there were four adult alpaca females, and all of them were included in the experiment. The farms were as follows:

- Alpaca Farm of Mr. Radek Vaněk in Líska near Česká Kamenice,
- Alpakafarma.cz of Mrs. Eleonora Hlaváčová in Veská near Pardubice,
- Farm of Mrs. Jana Dufková in Zámostí-Blata near Jičín.

The number of twelve females was considered as sufficient for this research. In alpacas and other lamoids, experiments focused on measuring hormone levels were often done with groups with even less than ten females (Volkery et al., 2012).

All animals were in their reproductive period, which means that they were at least one year old (Fernández-Baca, 1993). Six alpacas have already had young ones in the past

and five females were still heifers. In one female, no information about previous pregnancies was obtained from the breeder. Distinguishing individual females from each other was possible in spite of the existence of females of the same colour, because of unique features of each animal. Detailed information about the animals is provided in Table 1, for photos of all animals see Annexe 2.

Table 1: Detailed information about twelve female alpacas included in the research

Farm	Animal Name	Date of birth	Heifer	Health status
Alpaca Farm	Kumasi	01/01/2004	no data	poor (parasitosis[1])
	Minea	10/07/2011	yes	good
	Morena	12/06/2008	no	good
	Tonja	14/06/2008	no	poor (parasitosis[1])
Alpakafarma.cz	Aranka	13/07/2011	yes	excellent
	Ema	02/05/2005	no	good (persisting CL)
	Kapička	24/06/2011	yes	excellent
	Popelka	03/07/2007	no	good (lack of zinc)
Farm of Mrs. Dufková	Alina	23/08/2010	yes	excellent
	Blanka	10/04/2010	no	good
	Karlottka	14/09/2010	yes	excellent
	Rozárka	01/08/2010	no	excellent

[1] The parasitosis was not specified by the breeder. However, the health status of animals was serious and they were treated for some time period with antibiotics.

The diet of animals was very similar in all farms. It was based on the combination of hay and pasture, supplemented by concentrated feed for llamas and alpacas produced by the manufacturer Výroba krmných směsí - Sehnoutek a synové v.o.s. (Voleč, Lázně Bohdaneč). The composition and recommended dosage of this compound feed for alpacas is available in Annexe 3. Fresh water was provided *ad libitum*.

Breeding management was a little different among farms. On Alpaca Farm of Mr. Vaněk and farm of Mrs. Dufková, modified pasture breeding was mainly used. Female(s) spent a few days together with an individual male and the exact date of mating was sometimes not known. On Alpakafarma.cz of Mrs. Hlaváčová, hand breeding was used. Females were introduced to a male individually for approximately one hour. This was repeated after a few days. If females did not reject mating during the second attempt of mating, they were introduced to the male once more.

The date of the last successful mating as a day of probable conception and the date of expected parturition were obtained from breeders. The pregnancy of animals that gave birth during the research period was confirmed by the parturition of the offspring that came true. The dates of parturition were recorded. Pregnancy of females, which did not give birth during the research period, was confirmed by a veterinarian by means of P4 blood level tests.

4.3 Sample collection

The test of fresh urine sampling was carried out on the 7th March 2013. Then the samples were collected repeatedly in 6-8 week intervals from April 2013 to February 2014. Five collections of urine samples were planned on each of the three farms during this period.

The urine was caught to the half-litre plastic cups that were fastened on a telescopic rod (Haberová et al., 2011; Haberová et al., 2012) during spontaneous urination of an animal, see Figures 1, 2 and 3. The methods used for inducing urination were only indirect and non-invasive. Cleaning communal dung piles, driving the animals over them and spillage of urine of another alpaca on the communal dung pile were exercised.

After collection, the urine was poured into small plastic containers (volume of 20 ml) for the transportation of urine, see Figure 3. Detailed information about urine samples is provided in Tables 2 and 3, but some information was not possible to obtain from one breeder – Mr. Vaněk.

Figure 2: Urine collecting (photo: R. Vaněk)

Figure 3: Detail of collecting (photo: author)

Table 2: Detailed information about urine samples and the reproduction status of alpaca females during the research period

Farm	Animal	Number of samples during		Date of		Pregnancy confirmation
		pregnancy	non-pregnancy	last mating before parturition	parturition[2]	
Alpaca Farm	Kumasi	0	5	non-pregnant	non-pregnant	non-pregnant
	Minea	2	3	missing data	02/08/2013	parturition date
	Morena[1]	4	1	missing data	17/08/2013-13/10/2013[3]	parturition date
				31/10/2013	(11/10/2014)	missing data
	Tonja	3	2	missing data	17/08/2013-13/10/2013[3]	parturition date
Alpakafarma.cz	Aranka	3	2	09/2013	(08-09/2014)	P4 blood test (30/10/2013)
	Ema	0	5	non-pregnant	non-pregnant	non-pregnant
	Kapička	5	0	23/06/2013	(03/06/2014)	P4 blood test (30/10/2013)
	Popelka	0	5	non-pregnant	non-pregnant	non-pregnant
Farm of Mrs. Dufková	Alina	4	1	11/06/2013	(22/05/2014)	P4 blood test (05/10/2013)
	Blanka	0	5	non-pregnant	non-pregnant	non-pregnant
	Karlottka	3	2	07/08/2012	11/09/2013	parturition date
	Rozárka	4	1	11/06/2013	(22/05/2014)	P4 blood test (05/10/2013)
In total	12	28	32			

[1] Morena was the only female that was pregnant on the beginning of the research period, gave birth during the research period and became pregnant again before the end of the research period.

[2] In case of future parturition, the expected date is given in parenthesis.

[3] More precise data were impossible to obtain.

Table 3: Dates of all collections of urine on the three farms in different seasons

Farm	Spring	Summer	Autumn	Winter
Alpaca Farm	30/04/2013	25/06/2013 17/08/2013	13/10/2013 24/11/2013	
Alpakafarma.cz		21/07/2013	01/09/2013 20/10/2013	09/12/2013 09/02/2014
Farm of Mrs. Dufková	13/05/2013	02/07/2013 26/08/2013	14/10/2013 25/11/2013	

4.4 Sample processing

Immediately after the sampling, pH was tested by Duotest® double zone pH-indicator papers (Macherey-Nagel GmbH & Co. KG, Germany) with measuring range of pH 7.0-10.0.

Also, the information about specific gravity, pH again, presence of leucocytes, nitrite, protein, glucose, ketones, urobilinogen, bilirubin, blood and haemoglobin, was tested by diagnostic test strips for urinalysis DekaPhan® Leuco (Erba Lachema s.r.o., Czech Republic) at this moment. This information was described just for 56 samples, because it has been recorded during the second collection of samples for the first time. The only exception is specific gravity, which was measured in all samples. The equipment for these analyses is presented in Figure 4. All these measurements were considered as approximate, because diagnostic test strips for urinalysis DekaPhan® Leuco were designated for human use, and because urine samples could be contaminated by faeces during sampling.

Figure 4: Equipment for collecting, transportation and storage of samples – a telescopic rod, plastic cups and containers (photo: author)

Figure 5: Equipment for urinalysis – Duotest® pH-indicator papers and test strips for urinalysis DekaPhan® Leuco (photo: author)

In case of the seed germination test, the samples from eleven collections were processed on the same day or the day after sampling. There were just four exceptions, when the experiments started two, three, seventeen and nineteen days after sampling. The barium chloride test and the Cuboni reaction were done with some delay. The urine samples were submitted to the barium chloride test within one week after collection of samples. In case of the Cuboni reaction, samples were transported to the SVI Prague within one week and then processed within 20 days at maximum. In the meantime, the samples were stored in a refrigerator (Haberová et al., 2011) at the temperature of 5-7 °C. Before testing, the urine was kept outside the refrigerator to reach the room temperature and then it was homogenized.

The seed germination tests were done under laboratory conditions. The barium chloride tests were carried out in the laboratory of the Department of Animal Science and Food Processing, FTA, CULS Prague. Cuboni reactions were done in the SVI Prague, where this method is standardized for mares.

4.4.1 Seed germination test

For the seed germination test, the urine was diluted with distilled water in the ratio 1:4 (Veena and Narendranath, 1993; Dilrukshi and Perera, 2009; Krishna Rao and Veena, 2009; Rao Krishna and Veena, 2009; Narayana Swamy et al., 2010) and 1:14 (Dilrukshi and Perera, 2009). 15 ml of the solution was applied on 50 seeds (Dilrukshi and Perera, 2009; Narayana Swamy et al., 2010) in sterile Petri dishes (Krishna Rao and Veena, 2009; Narayana Swamy et al., 2010) marked with the abbreviated name of each animal and urine concentration.

Wheat seeds (Veena and Narendranath, 1993; Krishna Rao and Veena, 2009; Rao Krishna and Veena, 2009; Narayana Swamy et al., 2010) and mung bean (*Vigna radiata*) seeds (Dilrukshi and Perera, 2009; Rao Krishna and Veena, 2009) were used for the testing, see Figure 5. The seeds that were used for testing were in bio quality intended for human consumption. They were purchased in health food stores. Mung bean seeds of the product line Albert Bio (Ahold Czech Republic, a.s.) were used. Together with mung beans, spelt (*Triticum spelta*) of the brand Pro-Bio (Pro-Bio, s.r.o., Czech Republic) was used for the processing of the seed germination test in 12 samples from the first three collections of urine (2 collections on Alpaca Farm of Mr. Vaněk and 1 collection on the farm of Mrs. Dufková). This kind of seeds was considered as not suitable for the seed

germination test processing, because the seeds usually did not germinate even in distilled water. Because of this, the results of the seed germination test from the first three collections were not used in analyses. For the rest of the samples, winter wheat (*Triticum aestivum*) of the brand Country Life (Country Life, s.r.o., Czech Republic) was successfully used.

There were always eight Petri dishes for each urine sample. Four Petri dishes contained wheat seeds and four contained mung beans. From the four Petri dishes with wheat seeds, two contained urine diluted in the ratio 1:4, and two contained urine diluted in the ratio 1:14. The same 2 + 2 setting was applied to Petri dishes with mung beans.

Each time, control test was also done separately for 50 wheat seeds and 50 mung beans just with 15 ml of distilled water (Veena and Narendranath, 1993; Dilrukshi and Perera, 2009; Krishna Rao and Veena, 2009; Rao Krishna and Veena, 2009; Narayana Swamy et al., 2010).

The number of germinated seeds was counted (Veena and Narendranath, 1993; Dilrukshi and Perera, 2009; Krishna Rao and Veena, 2009; Rao Krishna and Veena, 2009; Narayana Swamy et al., 2010) in 24 hour intervals on five consecutive days. The first counting was performed 24 hours after the start of the experiment and every subsequent counting followed 24 hours after the previous one.

The length of shoots was measured (Dilrukshi and Perera, 2009; Rao Krishna and Veena, 2009) in millimetres by a ruler on the fifth day (Veena and Narendranath, 1993; Narayana Swamy et al., 2010), see Figure 6 and Annexe 4.

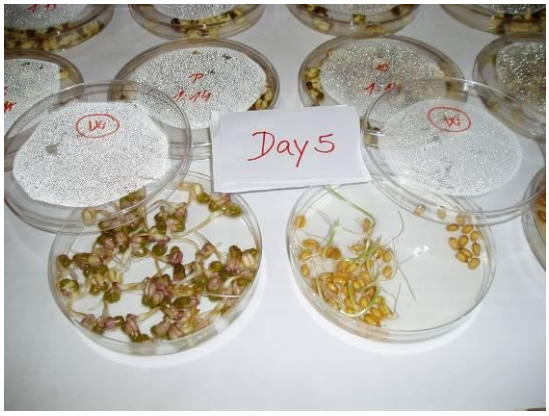

Figure 6: Water control tests on the 5th day – mung beans on the left and winter wheat seeds on the right (photo: author)

Figure 7: Shoot length measuring on the 5th day – mung beans on the left and winter wheat seeds on the right (photo: author)

4.4.2 Barium chloride test

For the barium chloride test, 5 ml of urine was poured into the test tube and mixed with 5 ml of 1% solution of $BaCl_2$ (Krishna Rao and Veena, 2009; Lalrintluanga and Dutta, 2009), see Figure 7. The solution was then agitated and let in a test tube to stand for 5 minutes (Ndu et al., 2000a; Ndu et al., 2000b). Then the results were evaluated. "Urine samples which showed any degree of cloudiness or turbidity after 5 minutes were regarded as having shown precipitate with the reagent" (Ndu et al., 2000a; Ndu et al., 2000b), see Figure 8.

Figure 8: Barium chloride test processing (photo: author)

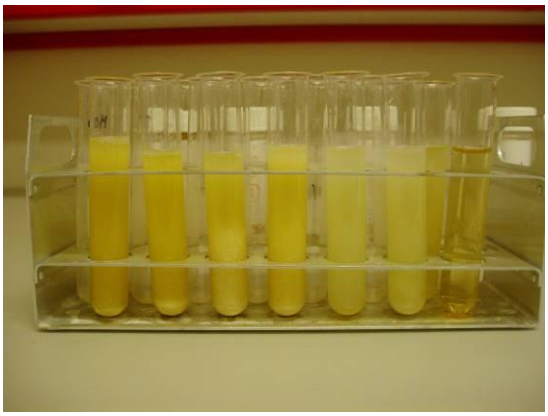

Figure 9: Barium chloride test – the only clear solution is on the right (photo: author)

4.4.3 Cuboni reaction

The urine for the Cuboni reaction was filtrated through the filter paper. 5 ml of filtrated urine was mixed with 1 ml of concentrated hydrochloric acid (HCl) in the test tube. Then the test tube was put into the boiling water bath for 10 minutes. After cooling, 6 ml of toluene was added and the solution was stirred properly. After one minute, two layers of the solution were divided. The lower layer with toluene was separated and filtrated to the clear test tube containing 1 ml of concentrated H_2SO_4. The mixture was stirred properly again and put into the water bath (with the temperature of 80 °C) for approximately 15 minutes. After cooling, the colour of opalescence in the test tube placed on the dark background was evaluated.

The photos of the reaction are presented in Annexe 5 and the example of protocol with results of the Cuboni reaction is in Annexe 6.

4.5 Accuracy evaluation

In the seed germination test, it was tested if the seeds treated with urine of pregnant females would have lower germination rates (Krishna Rao and Veena, 2009) and shorter shoots than seeds in urine of non-pregnant females (Veena and Narendranath, 1993; Dilrukshi and Perera, 2009; Rao Krishna and Veena, 2009; Narayana Swamy et al., 2010). During barium chloride processing, the solution should have stayed clear in pregnant animals while in non-pregnant animals white precipitation should have occurred (Ndu et al., 2000a; Ndu et al., 2000b; Krishna Rao and Veena, 2009; Lalrintluanga and Dutta, 2009). In case of urine of pregnant females, green opalescence should be detected after the Cuboni reaction. In urine of non-pregnant animals, brown-red colouring should be present (Čadová, 2013).

The accuracy of the seed germination test, the barium chloride test, and the Cuboni reaction was determined by comparison of their results with the real reproduction status of the animals. The status was either pregnant or non-pregnant, but the reliability of individual tests in pregnant females was counted not only for the whole pregnancy period, but also for its halves and thirds.

The information about the exact phase of pregnancy at the time of sampling was counted retrospectively from the date of parturition. In females that did not give birth during the research period, the date of the last successful mating plus 345 days as the mean length of pregnancy (Volkery et al., 2012) were used for estimation of parturition time.

The influence of the season on the accuracy of tests was assessed. The research period was divided into four seasons – spring (March, April, May), summer (June, July, August), autumn (September, October, November), and winter (December, January, February). The impact of the season on seed germination rates and shoot lengths was assessed on control samples. In shoot lengths, comparisons of the relations between shoot lengths of seeds treated with urine of pregnant and non-pregnant females were also used for evaluation of the influence of the season. Differences in results of the barium chloride test and the Cuboni reaction among seasons were tested in urine samples of non-pregnant females.

The influence of female Ema that suffered from persisting CL was assessed in the urinalysis, the barium chloride test and the Cuboni reaction. It was because the urine of this whole research period-non-pregnant female could act like the urine of pregnant female in these analyses. The same influence of this female was not considered in the seed

germination test, because it is known that the P4 had no effect on seed germination rates and shoot lengths (Nirmala et al., 2008).

For evaluating the reliability of the seed germination test as pregnancy diagnostic test in alpacas, just germination rates of seeds and lengths of their shoots in urine of pregnant and non-pregnant females were assessed. The controls were not included in these analyses.

In seed germination rates counting, the maximal possible germination rate was 100 seeds and the results were provided as numbers of germinated seeds. In case of shoot lengths measuring, the results were provided in mm of shoot length.

4.6 Data analysis

Data were statistically evaluated in the StatisticaCz 12 program (StatSoft, Inc., 2013). For all calculations significance level $\alpha = 0.05$ was established. All calculated numerical values were rounded off to two decimal places. The results were expressed as mean ± SEM.

For analyses, non-parametric tests were used because the data have not had the normal distribution (Kolmogorov-Smirnov test, $p < 0.05$). The only exception was the evaluation of shoots lengths in the seed germination test. In total, 18,429 measurements of shoot length were obtained and it was decided to use parametric tests for these analyses.

To analyse the data, following non-parametric tests were used: Kruskal-Wallis test, Mann-Whitney U test, Pearson's chi-squared test, Wilcoxon signed ranks test. For evaluation of data with shoot lengths, unpaired (two-sample) t-test and one-way ANOVA were used.

5. Results

5.1 Sample collection

In total, 60 urine samples from both non-pregnant and pregnant females in different stages of pregnancy were collected on three farms during the research period. There was no problem with the urine collection in the majority of females (75%). Three females (25%) were a little bit timid and the collection was time consuming and seemed to be impossible in them. However, the urine sample was always obtained from them after some time, although occasionally the amount of urine was not sufficient to perform all pregnancy tests. In problematic females, no improvement in accustomation to sampling was observed during the research.

The methods used for inducing urination appeared to be successful in alpaca females. The mean time needed to get one urine sample from one female was approximately one hour.

5.2 Urinalysis

Mean value of pH measured by DekaPhan® Leuco test strips (n = 60) was 8.35 ± 0.10, the range was between 6.00 and 9.00. The average value of pH measured by Duotest® double zone pH-indicator papers (n = 52) was 8.33 ± 0.06, the range was between 7.00 and 8.80.

No significant difference was found between testing of pH by diagnostic test strips DekaPhan® Leuco and Duotest® double zone pH-indicator papers (Wilcoxon signed ranks test: T = 356.00, Z = 1.62, p = 0.10).

In case of pH measuring by DekaPhan® Leuco test strips, 54 samples (90%) were alkaline, 3 samples (5%) were neutral, and 3 samples were acid (5%). In case of pH measuring by Duotest® double zone pH-indicator papers, 50 samples (96.15%) had alkaline pH values and 2 samples (3.85%) were neutral.

In urinary pH values, no significant difference was found among farms, no matter if it was measured by diagnostic test strips DekaPhan® Leuco (Kruskal-Wallis test: H (2, N = 60) = 2.14, p = 0.34) or Duotest® double zone pH-indicator papers (Kruskal-Wallis test: H (2, N = 52) = 3.95, p = 0.14).

No significant difference was found between urinary pH of pregnant and non-pregnant females, no matter if it was measured by diagnostic test strips DekaPhan® Leuco (Mann-Whitney U test: U = 395.00, Z = 0.78, p = 0.44) or Duotest® double zone pH-indicator papers (Mann-Whitney U test: U = 277.00, Z = 1.07, p = 0.28). The results were similar even if the samples from female Ema were not included in analyses ($p > 0.05$).

The specific gravity of urine measured by DekaPhan® Leuco (n = 60) was 1.000 in 36 samples (60.00%), 1.005 in 21 samples (35.00%), 1.010 in 2 samples (3.33%) and 1.015 just in 1 sample (1.67%). The average value of specific gravity of alpacas' urine was 1.00 ± 0.00.

Leucocytes (10-25 Leu/μl) were detected just in 1 urine sample (1.79%) of the animal suffering from parasitosis, namely Kumasi. Nitrites (light positive) were found in 2 urine samples (3.57%) that were collected on the farm of Mrs. Dufková. Protein occurred in 35 samples (62.50%) that came from all three farms. The content of protein was as follows: 0.3 g/l (30 mg/dl) in 24 samples (42.86%), 1.0 g/l (100 mg/dl) in 10 samples (17.86%), and 5.0 g/l (500 mg/dl) in 1 sample (1.79%). All samples were negative for glucose. Ketones (1.5 mmol/l = 16 mg/dl) were found just in 2 samples (3.57%) that were collected on the Alpakafarma.cz of Mrs. Hlaváčová. Higher levels of urobilinogen, 17 μmol/l (1 mg/dl), were detected in 48 samples (85.71%), and bilirubin (+) was present in 52 samples (92.86%). Blood occured in 2 samples (3.57%) of two females from the farm of Mrs. Dufková. The content of erythrocytes was 5-10 Ery/μl in 1 sample (1.79%), and 50 Ery/μl also in 1 sample (1.79%).

5.3 Seed germination rates counting

In total, germination rates were counted in 22,950 seeds. In control samples, the number was 1,350 seeds (750 mung beans; 600 winter wheat seeds). 11,800 seeds (6,400 mung beans; 5,400 winter wheat seeds) treated with urine of non-pregnant females were counted. The number in pregnant females was 9,800 seeds (5,600 mung beans; 4,200 winter wheat seeds).

5.3.1 Influence of the season on control germination rates

From the 1st to the 5th day of germination rates counting, no significant difference in numbers of germinated control mung beans was found among seasons ($p > 0.05$).

The same results were obtained in control wheat seeds from the 2nd to the 5th day of counting ($p > 0.05$). In contrast, significant difference in germination rates of wheat seeds was found among seasons on the 1st day (Kruskal-Wallis test: $H (2, N = 12) = 6.83$, $p = 0.03$). Germination rates in summer (85.00 ± 0.58) were significantly higher than the rates in autumn (Multiple comparison test: $p = 0.04$, 65.67 ± 5.43).

Based on these results, all data with germination rates of mung beans from different seasons were assessed together. The same was done with the data of wheat seeds, but the data from the 1st measuring day of wheat seeds were assessed separately for individual seasons.

5.3.2 Pregnancy diagnosis by seed germination rates counting

5.3.2.a Mung beans and urine with 1:4 concentration

Germination rates of mung beans in urine of pregnant females with 1:4 concentration were significantly higher than germination rates of beans treated with urine of non-pregnant females during the first three days of the experiment (Mann-Whitney U test: $p < 0.05$, see Table 4). In the 4th and 5th day, no significant difference was found between them ($p > 0.05$).

Table 4: Results of comparison of germination rates of mung beans in urine with 1:4 concentration of pregnant and non-pregnant females from the 1st to the 3rd day

Day of counting	Result of Mann-Whitney U test		
	U	**Z**	**p**
1st	208.00	-2.36	0.02
2nd	292.00	-2.15	0.03
3rd	282.00	-2.12	0.03

In case of dividing the pregnancy into halves, significant difference in germination rates of mung beans was found among the three groups: 1:4 diluted urine of non-pregnant females and 1:4 diluted urine of pregnant females in the 1st and the 2nd half of pregnancy from the 1st to the 4th day of counting (Kruskal-Wallis test: $p < 0.05$, see Table 5).

Table 5: Results of the differences in germination rates of mung beans in 1:4 diluted urine of non-pregnant females and females in the 1st and 2nd half of pregnancy from the 1st to the 4th day of counting

Day of counting	Result of Kruskal-Wallis test		
	N	H	p
1st	52	7.40	= 0.02
2nd	59	6.49	= 0.04
3rd	58	8.34	= 0.02
4th	59	6.12	< 0.05

The difference was caused by significantly higher germination rates of beans treated with urine of females in the 2nd half of pregnancy than germination rates of beans treated with urine of non-pregnant females (Multiple comparison test: $p < 0.05$). However, germination rates of beans treated with urine of females in the 2nd half of pregnancy or non-pregnant females did not differ significantly from germination rates of beans treated with urine of females in the 1st half of pregnancy ($p > 0.05$). On the 5th day, no significant difference was found among all three groups (Kruskal-Wallis test: $H (2, N = 60) = 5.26$, $p = 0.07$). Details of these comparisons are presented in Figure 9.

In case of dividing the pregnancy into thirds, the groups (1:4 diluted urine of non-pregnant females and 1:4 diluted urine of pregnant females in the 1st, 2nd and 3rd third of pregnancy) did not differ significantly from each other in germination rates of mung beans during all five days ($p > 0.05$).

5.3.2.b Mung beans and urine with 1:14 concentration

Germination rates of mung beans in urine with 1:14 concentration were not significantly different between pregnant and non-pregnant females ($p > 0.05$), even if the pregnancy was divided into halves ($p > 0.05$) or thirds ($p > 0.05$) during all five days of the experiment.

The only exception was the 2nd day of counting in case of dividing the pregnancy into halves, where the only significant difference among groups was found (Kruskal-Wallis test: $H (2, N = 60) = 8.73$, $p = 0.01$). In this case, germination rate of beans placed in the urine of females in the 2nd half of pregnancy was significantly higher (98.67 ± 0.66), than these in the urine of other two groups – non-pregnant females (Multiple comparison test: $p = 0.02$, 95.19 ± 0.96) and females in the 1st half of pregnancy (Multiple comparison test: p

< 0.05, 95.38 ± 1.16). Germination rates of non-pregnant females and females in the 1st half of pregnancy were not significantly different on the 2nd day ($p > 0.05$).

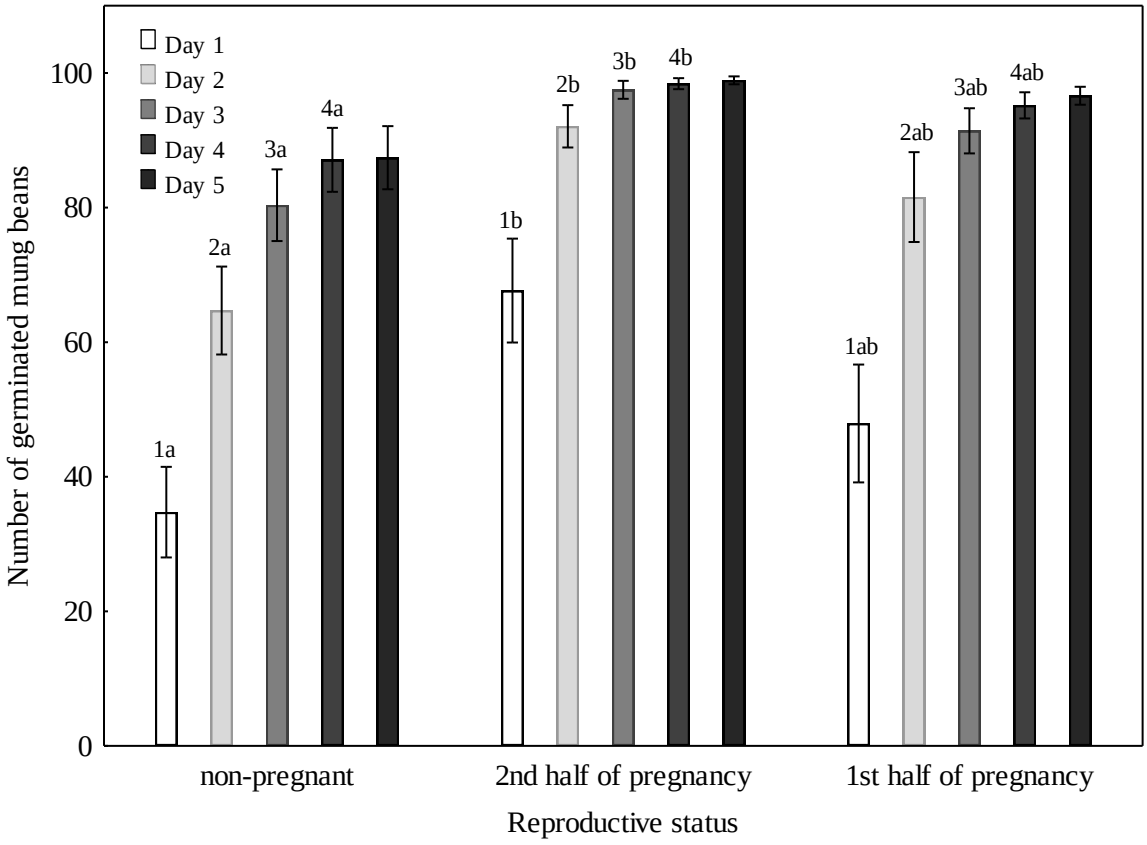

Figure 9: Numbers of germinated mung beans in urine of non-pregnant females and pregnant females in the 1st and 2nd half of pregnancy with 1:4 concentration during five days of the experiment. Data are presented as mean ± SEM. Different letters present significant differences among groups (Multiple comparison test: $p < 0.05$).

5.3.2.c Wheat seeds and urine with 1:4 and 1:14 concentration

No significant difference in germination rates of wheat seeds was found between the urine of pregnant and non-pregnant females ($p > 0.05$), even if the pregnancy was divided in halves ($p > 0.05$) or thirds ($p > 0.05$). No matter, if the concentration of the urine was 1:4 or 1:14.

5.3.3 General influence of urine on seed germination rates

In non-pregnant females, germination rates of mung beans treated with urine with 1:4 concentration were significantly lower than the rates of mung beans treated with urine

with 1:14 concentration from the 1st to the 5th day of counting (Mann-Whitney U test: $p < 0.05$, see Table 6). In pregnant females, the same results were obtained from the 2nd to the 5th day (Mann-Whitney U test: $p < 0.05$, see Table 6). However, no significant difference was found between germination rates of beans treated with different concentrations on the 1st day in pregnant females ($p > 0.05$).

Germination rates of wheat seeds treated with urine with 1:4 concentration were always significantly lower than the rates of wheat seeds treated with urine with 1:14 concentration, no matter if it was urine of pregnant or non-pregnant females (Mann-Whitney U test: $p < 0.05$, see Table 7).

Table 6: Results of comparison of the effect of two urine concentrations on germination rates of mung beans in non-pregnant (1st – 5th day) and pregnant females (2nd – 5th day)

Urine of	Day of counting	Result of Mann-Whitney U test		
		U	Z	p
Non-pregnant females	1st	195.50	-2.92	< 0.01
	2nd	249.50	-3.38	< 0.01
	3rd	223.50	-3.48	< 0.01
	4th	286.50	-2.87	< 0.01
	5th	314.00	-2.65	= 0.01
Pregnant females	2nd	209.50	-2.98	< 0.01
	3rd	227.00	-2.37	= 0.02
	4th	265.00	-2.07	= 0.04
	5th	268.00	-2.02	= 0.04

Table 7: Results of comparison of the effect of two urine concentrations on germination rates of wheat seeds in non-pregnant and pregnant females (from the 1st to the 5th day)

Urine of	Day of counting	Result of Mann-Whitney U test		
		U	Z	p
Non-pregnant females	1st	108.00	-3.13	< 0.01
	2nd	163.00	-3.34	< 0.01
	3rd	166.00	-3.28	< 0.01
	4th	157.50	-3.43	< 0.01
	5th	154.50	-3.62	< 0.01
Pregnant females	1st	79.50	-2.59	= 0.01
	2nd	83.50	-3.43	< 0.01
	3rd	82.00	-3.47	< 0.01
	4th	74.50	-3.66	< 0.01
	5th	77.00	-3.60	< 0.01

5.3.3.a Mung beans and urine with 1:4 concentration

In the period between the 1st and the 5th day, significant difference in germination rates of mung beans was found among the three groups: control samples, 1:4 diluted urine of pregnant females, and 1:4 diluted urine of non-pregnant females (Kruskal-Wallis test: $p < 0.05$, see Table 8). For detailed information, see Figure 10.

5.3.3.b Mung beans and urine with 1:14 concentration

In the 1st and 2nd day of counting, significant difference in germination rates of mung beans was found among the three groups: control samples, 1:14 diluted urine of pregnant females, and 1:14 diluted urine of non-pregnant females (Kruskal-Wallis test: $p < 0.05$, see Table 8). This was caused by significantly higher germination rates of beans in controls (87.87 ± 4.30 on the 1st day, 99.20 ± 0.26 on the 2nd day) than in urine of non-pregnant females (59.07 ± 5.70 on the 1st day, 95.19 ± 0.96 on the 2nd day). On the 3rd, 4th and 5th day, no significant difference in germination rates of beans was found among all three groups ($p > 0.05$).

Table 8: Results of the analysis of differences in germination rates of mung beans among controls, urine of pregnant and non-pregnant females (from the 1st to the 5th day in case of urine with 1:4 concentration, the 1st and 2nd day in case of urine with 1:14 concentration)

Concentration of urine	Day of counting	Result of Kruskal-Wallis test		
		N	H	p
1:4	1st	67	25.19	< 0.01
	2nd	74	23.32	< 0.01
	3rd	73	18.21	< 0.01
	4th	74	12.42	< 0.01
	5th	75	7.29	= 0.03
1:14	1st	67	9.38	< 0.01
	2nd	75	12.23	< 0.01

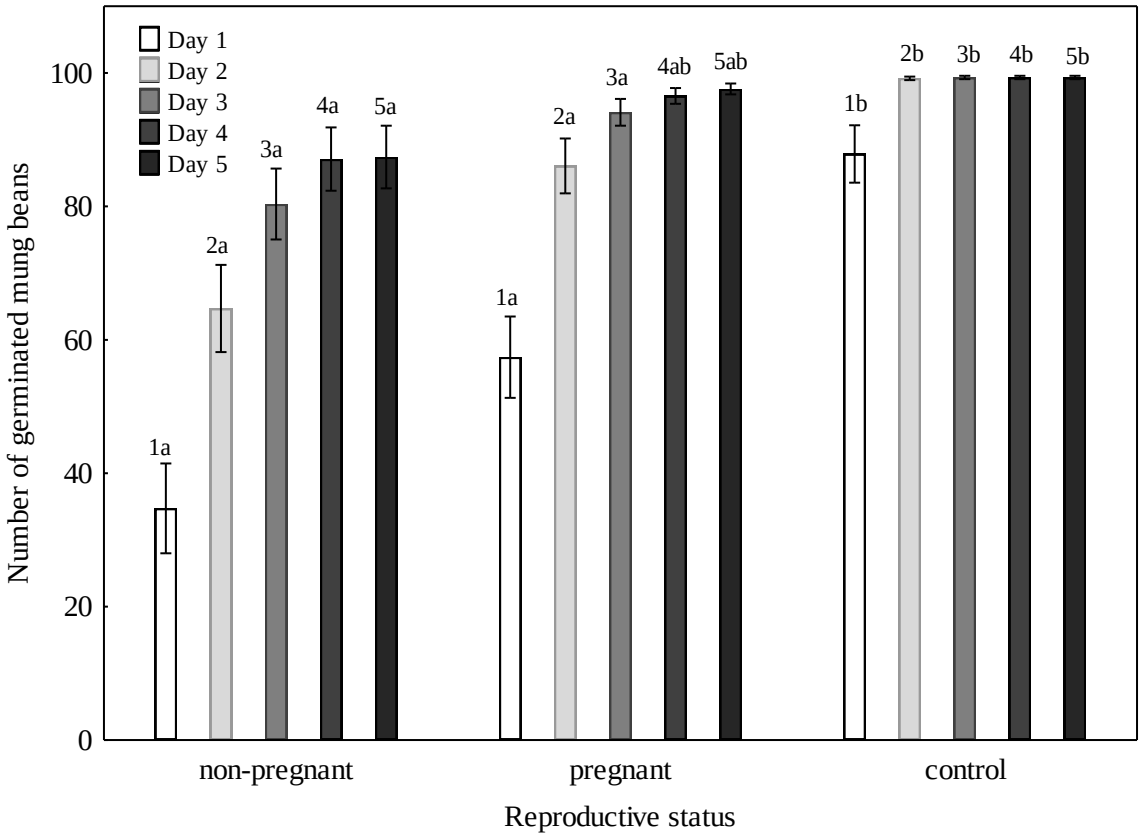

Figure 10: Numbers of germinated mung beans in controls and urine of pregnant and non-pregnant females with 1:4 concentrations during the five days of the experiment. Data are presented as mean ± SEM. Different letters present significant differences among groups (Multiple comparison test: $p < 0.05$).

5.3.3.c Wheat seeds and urine with 1:4 concentration

In case of the 1st day, significant difference in germination rates of wheat seeds was found among control samples, 1:4 diluted urine of pregnant females, and 1:4 diluted urine of non-pregnant females in summer (Kruskal-Wallis test: $H (2, N = 20) = 10.21$, $p = 0.01$) and in autumn (Kruskal-Wallis test: $H (2, N = 22) = 6.98$, $p = 0.03$). However, no significant difference among groups was found in winter ($p > 0.05$).

During the summer period, germination rates of controls (85.00 ± 0.58) were significantly higher than the rates of wheat seeds treated with 1:4 diluted urine of pregnant (Multiple comparison test: $p = 0.03$, 24.22 ± 9.70) and non-pregnant females (Multiple comparison test: $p = 0.01$, 12.14 ± 7.68). Germination rates of seeds in urine of pregnant and non-pregnant females did not differ significantly in summer ($p > 0.05$). In autumn, a difference was found between controls and urine of pregnant females but there was a trend to no significant difference among all groups (Multiple comparison test: $p < 0.05$).

From the 2nd to the 5th day of counting, significant difference in germination rates was found among groups (Kruskal-Wallis test: $p < 0.05$, see Table 9). Germination rates of controls were significantly higher than germination rates of seeds treated with 1:4 diluted urine, no matter if it was urine of pregnant (Multiple comparison test: $p < 0.05$) or non-pregnant females (Multiple comparison test: $p < 0.05$). However, germination rates of seeds in urine of pregnant and non-pregnant females did not differ from each other significantly for all four days ($p > 0.05$).

5.3.3.d Wheat seeds and urine with 1:14 concentration

On the 1st day, significant difference in germination rates was found among groups (control samples, 1:14 diluted urine of pregnant females, and 1:14 diluted urine of non-pregnant females) in summer (Kruskal-Wallis test: $H (2, N = 20) = 9.26$, $p = 0.01$) and winter (Kruskal-Wallis test: $H (2, N = 10) = 6.33$, $p = 0.04$). However, there was no significant difference among groups in autumn (Kruskal-Wallis test: $H (2, N = 22) = 2.40$, $p = 0.30$). In summer, the situation was the same as with wheat seeds in 1:4 urine concentration – germination rates of controls (85.00 ± 0.58) were significantly higher than the rates of wheat seeds treated with 1:14 diluted urine of pregnant (Multiple comparison test: $p = 0.02$, 53.44 ± 10.70) and non-pregnant females (Multiple comparison test: $p = 0.02$, 48.71 ± 13.29). No significant difference in germination rates between urine of pregnant and non-pregnant females was found on the 1st day in summer ($p > 0.05$). In winter, the only significant difference was between controls and urine of non-pregnant females (Multiple comparison test: $p = 0.04$).

In the period from the 2nd to the 5th day, the results of comparison of germination rates among groups were very similar to results with wheat seeds and 1:4 diluted urine from the same days (Kruskal-Wallis test: $p < 0.05$, see Table 9). On these days, germination rates of controls were significantly higher than germination rates of seeds treated with 1:14 diluted urine of pregnant (Multiple comparison test: $p < 0.05$) and non-pregnant females (Multiple comparison test: $p < 0.05$). In the same time, germination rates of seeds in urine of pregnant and non-pregnant females did not differ from each other significantly for all four days ($p > 0.05$).

Table 9: Results of the analysis of differences in germination rates of wheat seeds among controls, urine of pregnant and non-pregnant females (from the 2nd to the 5th day in urine with 1:4 concentration, and from the 2nd to the 5th day in urine with 1:14 concentration)

Concentration of urine	Day of counting	Result of Kruskal-Wallis test		
		N	H	p
1:4	2nd	59	24.60	< 0.01
	3rd	59	27.00	< 0.01
	4th	59	27.10	< 0.01
	5th	60	27.30	< 0.01
1:14	2nd	60	15.90	< 0.01
	3rd	60	17.00	< 0.01
	4th	60	17.10	< 0.01
	5th	60	16.90	< 0.01

5.4 Shoot lengths measuring

In total, lengths of shoots were measured in 18,429 seeds. In control samples, it was 2,520 seeds (1,490 mung beans; 1,030 winter wheat seeds). 8,443 shoot lengths of seeds (5,915 mung beans; 2,528 winter wheat seeds) treated with urine of non-pregnant females were measured. The number in pregnant females was 7,466 measurements (5,415 mung beans; 2,051 winter wheat seeds).

5.4.1 Influence of the season on shoot lengths in controls

Significant differences were found in shoot lengths of control mung beans among seasons (one-way ANOVA: $F(3, 740) = 293.52$, $p < 0.01$). Each season was significantly different from other seasons (Tukey's HSD test: $p < 0.01$). The longest shoots occurred in spring (72.07 ± 1.86), then in summer (53.20 ± 1.13), autumn (32.12 ± 0.86), and the shortest ones were measured in winter (16.58 ± 0.56).

In shoot lengths of control wheat seeds, significant differences among seasons were found too (one-way ANOVA: $F(2, 506) = 61.68$, $p < 0.01$). Wheat shoot lengths from summer (44.02 ± 2.82) were significantly longer than wheat shoot lengths from autumn (Tukey's HSD test: $p < 0.01$, 17.36 ± 1.57) and winter (Tukey's HSD test: $p < 0.01$, 10.20 ± 0.92). However, no significant difference was found between autumn and winter ($p > 0.05$) lengths of shoots.

Based on these results, the analyses of the differences between pregnant and non-pregnant females were done separately for individual seasons in mung beans. In wheat

seeds, summer data were assessed separately from autumnal and winter data that were assessed together.

5.4.2 Pregnancy diagnosis by shoot length measuring

5.4.2.a Mung beans and urine with 1:4 concentration

Significant difference was found between shoot lengths of mung beans treated with 1:4 diluted urine of pregnant and non-pregnant females in spring (unpaired t-test: $t = -6.54$, $df = 741$, $p < 0.01$), summer (unpaired t-test: $t = -6.20$; $df = 1{,}636$; $p < 0.01$) and autumn (unpaired t-test: $t = -5.82$; $df = 2{,}229$; $p < 0.01$). The shoot lengths of beans that were treated with urine of pregnant females (12.41 ± 0.33 in spring, 11.44 ± 0.36 in summer, 3.63 ± 0.05 in autumn) were always significantly longer than these in urine of non-pregnant females (9.74 ± 0.25 in spring, 7.82 ± 0.42 in summer, 3.25 ± 0.04 in autumn) in these seasons. In winter, the shoot lengths were not significantly different between pregnant and non-pregnant females ($p > 0.05$). Based on these results, reliability of the test for halves and thirds of pregnancy was assessed for the data from spring, summer, and autumn together. Data from winter were evaluated separately.

In spring, summer, and autumn, significant difference was found among these groups: non-pregnant females and pregnant females in the 1st and the 2nd half of pregnancy (one-way ANOVA: $F(2; 4{,}609) = 360.39$, $p < 0.01$). The shoot lengths of mung beans treated with 1:4 diluted urine of females in 2nd half of pregnancy were significantly longer (12.81 ± 0.31) than shoot lengths of beans treated with urine of non-pregnant females (Tukey's HSD test: $p < 0.01$, 5.36 ± 0.12) and females in the 1st half of pregnancy (Tukey's HSD test: $p < 0.01$, 5.91 ± 0.23). However, there was no significant difference between non-pregnant females and females in the 1st half of pregnancy ($p > 0.05$). In winter, shoot lengths did not significantly differ among all groups ($p > 0.05$).

In case of dividing pregnancy into thirds, significant difference was found among four groups (non-pregnant females, females in the 1st, 2nd, and 3rd third of pregnancy) in the period from spring to autumn (one-way ANOVA: $F(3; 4{,}213) = 47.14$, $p < 0.01$). The longest were the shoots of mung beans treated with 1:4 diluted urine of females in the 1st (7.27 ± 0.37) and 3rd (7.82 ± 0.29) third of pregnancy, and these two groups did not significantly differ from each other (Tukey's HSD test: $p > 0.05$). Middle shoot lengths occurred in urine of non-pregnant females (Tukey's HSD test: $p < 0.01$, 5.36 ± 0.12) and

the shortest shoot lengths were in urine of females in the 2nd third of pregnancy (Tukey's HSD test: $p < 0.01$, 3.78 ± 0.07). In winter, the groups were also significantly different in their shoot lengths (one-way ANOVA: $F(3, 756) = 6.73$, $p < 0.01$), but the relations among groups were different. Results of the Tukey's HSD test are available in Table 10.

Table 10: Results of the Tukey's HSD test for differences in shoot lengths of mung beans treated with 1:4 diluted urine of females in different reproductive statuses in winter (p-values in bold print mean significant difference), and the length of shoots of different groups

	shoot length	non-pregnant	1st third of pregnancy	2nd third of pregnancy	3rd third of pregnancy
non-pregnant	3.39 ± 0.02	—	p = 0.18	**p = 0.01**	p = 0.42
1st third of pregnancy	3.26 ± 0.03	p = 0.18	—	**p < 0.01**	**p = 0.03**
2nd third of pregnancy	3.56 ± 0.07	**p = 0.01**	**p < 0.01**	—	p = 0.83
3rd third of pregnancy	3.50 ± 0.00	p = 0.42	**p = 0.03**	p = 0.83	—

5.4.2.b Mung beans and urine with 1:14 concentration

Significant difference was found between shoot lengths of mung beans treated with 1:14 diluted urine of pregnant and non-pregnant females in all seasons – spring (unpaired t-test: $t = 3.74$, $df = 744$, $p < 0.01$), summer (unpaired t-test: $t = -6.30$; $df = 1{,}773$; $p < 0.01$), autumn (unpaired t-test: $t = -2.34$; $df = 2{,}368$; $p = 0.02$), and winter (unpaired t-test: $t = -4.62$; $df = 788$; $p < 0.01$). In spring, the shoot lengths of beans treated with urine of non-pregnant females (37.17 ± 0.65) were significantly longer than the shoot lengths of beans treated with urine of pregnant females (33.61 ± 0.69). In summer, autumn and winter, the results were opposite – the shoot lengths of beans treated with urine of non-pregnant females (29.30 ± 0.88 in summer, 9.42 ± 0.19 in autumn, 5.37 ± 0.12 in winter) were significantly shorter than these treated with urine of pregnant females (35.61 ± 0.57 in summer, 10.20 ± 0.27 in autumn, 6.24 ± 0.15 in winter). Based on these results, the reliability of the test for halves and thirds of the pregnancy was assessed for the data from summer, autumn, and winter together. Data from spring were evaluated separately.

In case of the dividing the pregnancy into halves, there were no females in the 1st half of pregnancy during spring. However, significant difference was found between non-pregnant females and females in the 2nd half of pregnancy (unpaired t-test: $t = 3.74$; $df =$

744; $p < 0.01$). Shoot lengths of mung beans treated with 1:14 diluted urine of non-pregnant females (37.17 ± 0.65) were significantly longer than shoot lengths of beans treated with urine of females in the 2nd half of pregnancy (33.61 ± 0.69).

In the period from summer to winter, significant differences were found among all three groups: non-pregnant females and females in the 1st and the 2nd half of pregnancy (one-way ANOVA: $F(2; 4{,}932) = 199.17$, $p < 0.01$). The longest were the shoots of beans treated with urine of females in the 2nd half of pregnancy (Tukey's HSD test: $p < 0.01$, 29.52 ± 0.79). In the middle were the shoots of beans treated with urine of females in the 1st half of pregnancy (Tukey's HSD test: $p < 0.01$, 18.03 ± 0.44) and the shortest shoot lengths had the beans treated with the urine of non-pregnant females (Tukey's HSD test: $p < 0.01$, 14.28 ± 0.32).

With dividing of pregnancy into thirds, there were just non-pregnant females and females in the 3rd third of the pregnancy in spring. The result were the same as with dividing the pregnancy into halves, because during this season, all pregnant females were in their 3rd third of the pregnancy.

From summer to winter, significant difference was found among four groups: non-pregnant females, females in the 1st, 2nd, and 3rd third of pregnancy (one-way ANOVA: $F(3; 4{,}833) = 196.09$, $p < 0.01$). The longest were the shoots of mung beans treated with 1:14 diluted urine of females in the 1st (24.76 ± 0.67) and 3rd (26.54 ± 0.84) third of pregnancy and these two groups did not significantly differ from each other (Tukey's HSD test: $p > 0.05$). Middle shoot lengths had the beans treated with the urine of non-pregnant females (Tukey's HSD test: $p < 0.01$, 14.28 ± 0.32), and the shortest shoot lengths had the beans treated with urine of females in the 2nd third of pregnancy (Tukey's HSD test: $p < 0.01$, 9.31 ± 0.25).

5.4.2.c Wheat seeds and urine with 1:4 concentration

In summer, significant difference was found between shoot lengths of wheat seeds treated with 1:4 diluted urine of pregnant and non-pregnant females (unpaired t-test: $t = -2.09$, $df = 501$, $p = 0.04$). The shoot lengths of seeds that were treated with urine of pregnant females (3.04 ± 0.43) were significantly longer than these in the urine of non-pregnant females (1.68 ± 0.17). During autumn and winter, there was also significant difference between these two groups (unpaired t-test: $t = -2.77$; $df = 1{,}080$; $p = 0.01$) and the shoot lengths of wheat seeds treated with urine of pregnant females (2.11 ± 0.14) were

again significantly longer then the shoot lengths of seeds treated with urine of non-pregnant females (1.77 ± 0.06). Based on these results, the reliability of the test for halves and thirds of the pregnancy was assessed for all these data together.

In case of the dividing the pregnancy into halves, there was a significant difference in the shoot lengths of wheat seeds among three groups: 1:4 diluted urine of non-pregnant females, females in the 1st and 2nd half of pregnancy (one-way ANOVA: $F(2; 1{,}582) = 7.90$, $p < 0.01$). The shoot lengths of wheat seeds treated with the urine of non-pregnant females (1.75 ± 0.05) were shorter than shoot lengths of seeds treated with urine of pregnant females, no matter if they were in the 1st (Tukey's HSD test: $p < 0.01$, 2.58 ± 0.31) or the 2nd (Tukey's HSD test: $p = 0.01$, 2.58 ± 0.31) half of pregnancy.

With dividing of pregnancy into thirds, shoot lengths of wheat seeds treated with 1:4 diluted urine of different groups (non-pregnant females, females in the 1st, 2nd, and 3rd third of pregnancy) differed significantly (one-way ANOVA: $F(3; 1{,}581) = 5.53$, $p < 0.01$). Results of the Tukey's HSD test are available in Table 11.

Table 11: Results of the Tukey's HSD test for differences in shoot lengths of wheat seeds treated with 1:4 diluted urine of females in different reproductive statuses (p-values in bold print mean significant differences), and length of shoots of different groups

	shoot length	non-pregnant	1st third of pregnancy	2nd third of pregnancy	3rd third of pregnancy
non-pregnant	1.75 ± 0.05	—	**p = 0.01**	p = 0.16	**p = 0.02**
1st third of pregnancy	2.76 ± 0.58	**p = 0.01**	—	p = 0.81	p = 0.96
2nd third of pregnancy	2.41 ± 0.21	p = 0.16	p = 0.81	—	p = 0.97
3rd third of pregnancy	2.58 ± 0.31	**p = 0.02**	p = 0.96	p = 0.97	—

5.4.2.d Wheat seeds and urine with 1:14 concentration

In summer, no significant difference was found between shoot lengths of wheat seeds treated with 1:14 diluted urine of pregnant and non-pregnant females ($p > 0.05$). The same results were obtained also from the data from autumn and winter ($p > 0.05$). Based on these results, the reliability of the test for halves and thirds of the pregnancy was assessed for data from all seasons together.

In case of the dividing the pregnancy into halves, there was a significant difference in the shoot lengths of wheat seeds among three groups: 1:14 diluted urine of non-pregnant

females, females in the 1st and 2nd half of pregnancy (one-way ANOVA: F (2; 2,992) = 4.92, p = 0.01). This significant difference was caused by longer shoots of seeds that were treated with the urine of females in the 2nd half of pregnancy (5.39 ± 0.63) than the shoot lengths of seeds treated with urine of non-pregnant females (Tukey's HSD test: p = 0.01, 3.85 ± 0.17). However, shoot lengths of seeds treated with urine of females in the 1st half of pregnancy (4.33 ± 0.32) did not differ significantly from both these two groups (p > 0.05).

With dividing of pregnancy into thirds, significant difference in shoot lengths of wheat seeds was found among four groups: 1:14 diluted urine of non-pregnant females, females in the 1st, 2nd, and 3rd third of pregnancy (one-way ANOVA: F (3; 2,991) = 4.61, p < 0.01). Results of the Tukey's HSD test are available in Table 12.

Table 12: Results of the Tukey's HSD test for differences in shoot lengths of wheat seeds treated with 1:14 diluted urine of females in different reproductive statuses (p-values in bold print mean significant differences), and length of shoots of different groups

	shoot length	non-pregnant	1st third of pregnancy	2nd third of pregnancy	3rd third of pregnancy
non-pregnant	3.85 ± 0.17	—	p = 0.12	p = 0.98	**p = 0.01**
1st third of pregnancy	4.79 ± 0.50	p = 0.12	—	p = 0.19	p = 0.73
2nd third of pregnancy	3.65 ± 0.29	p = 0.98	p = 0.19	—	**p = 0.03**
3rd third of pregnancy	5.39 ± 0.63	**p = 0.01**	p = 0.73	**p = 0.03**	—

5.4.3 General influence of urine on shoot lengths

In non-pregnant females, the shoot lengths of mung beans treated with urine with 1:4 concentration were always significantly shorter than the shoot lengths of mung beans treated with urine with 1:14 concentration (unpaired t-test: p < 0.05, see Table 13). In pregnant females, the same results were obtained in all seasons (unpaired t-test: p < 0.05, see Table 13).

In non-pregnant females, the shoot lengths of wheat seeds treated with urine with 1:4 concentration were significantly shorter than the shoot lengths of wheat seeds treated with urine with 1:14 concentration in both summer (unpaired t-test: t = -3.15, df = 556, p < 0.01), and autumn and winter (unpaired t-test: t = -10.41; df = 1,964; p < 0.01). In pregnant females, the same results were obtained in both summer (unpaired t-test: t = -

3.46; df = 1,023; $p < 0.01$), and autumn and winter (unpaired t-test: t = -4.34; df = 1,029; $p < 0.01$).

Table 13: Detailed results of comparison of the effect of two different urine concentrations on shoot lengths of mung beans in non-pregnant females and pregnant females in different seasons

Urine of	Season	Result of unpaired t-test		
		t	df	p
Non-pregnant females	spring	-39,57	793	$p < 0.01$
	summer	-19,79	1,292	$p < 0.01$
	autumn	-30,67	3,049	$p < 0.01$
	winter	-16,35	775	$p < 0.01$
Pregnant females	spring	-27,47	692	$p < 0.01$
	summer	-36,52	2,117	$p < 0.01$
	autumn	-23,33	1,548	$p < 0.01$
	winter	-17,95	771	$p < 0.01$

5.4.3.a Mung beans and urine with 1:4 concentration

Significant difference was found in shoot lengths of mung beans among three tested groups (control samples, 1:4 diluted urine of pregnant females, and 1:4 diluted urine of non-pregnant females) in all seasons. Concretely, it was in spring (one-way ANOVA: F (2, 840) = 2,434.7; $p < 0.01$), summer (one-way ANOVA: F (2; 1,881) = 1,342.4; $p < 0.01$), autumn (one-way ANOVA: F (2; 2,527) = 3,869.0; $p < 0.01$), and winter (one-way ANOVA: F (2, 856) = 1,930.3; $p < 0.01$). The relations among groups were similar in spring and summer and then in autumn and winter.

In spring and summer, the longest shoots had the beans in controls (72.07 ± 1.86 in spring, 53.20 ± 1.13 in summer). The middle length of shoots had the beans treated with urine of pregnant females (Tukey's HSD test: $p < 0.01$, 12.41 ± 0.33 in spring, 11.44 ± 0.36 in summer). The shortest were the shoot lengths of the beans treated with the urine of non-pregnant females (Tukey's HSD test: $p < 0.01$, 9.74 ± 0.25 in spring, 7.82 ± 0.42 in summer).

In autumn and winter, shoots of beans in controls were significantly longer (32.12 ± 0.86 in autumn, 16.58 ± 0.56 in winter) than the shoot lengths of beans treated with urine. No matter, if it was urine of pregnant (Tukey's HSD test: $p < 0.01$, 3.63 ± 0.05 in autumn, 3.47 ± 0.04 in winter) or non-pregnant females (Tukey's HSD test: $p < 0.01$, 3.25 ± 0.04 in

autumn, 3.39 ± 0.02 in winter). The shoot lengths of beans treated with urine of pregnant and non-pregnant females did not differ from each other during autumn and winter ($p > 0.05$).

5.4.3.b Mung beans and urine with 1:14 concentration

Among three tested groups (control samples, 1:14 diluted urine of pregnant females, and 1:14 diluted urine of non-pregnant females), significant difference in shoot lengths of mung beans was found in all seasons. Results for each season are provided: spring (one-way ANOVA: $F(2, 843) = 319.69$, $p < 0.01$), summer (one-way ANOVA: $F(2; 2{,}018) = 126.26$, $p < 0.01$), autumn (one-way ANOVA: $F(2; 2{,}666) = 878.12$, $p < 0.01$), and winter (one-way ANOVA: $F(2, 886) = 536.92$, $p < 0.01$).

In spring, controls had the longest shoots (72.07 ± 1.86), then were non-pregnant females (Tukey's HSD test: $p < 0.01$, 37.17 ± 0.65), and the shortest shoot lengths were in urine of pregnant females (Tukey's HSD test: $p < 0.01$, 33.61 ± 0.69).

In summer, controls had also the longest shoots (53.20 ± 1.13), but then were the shoots of pregnant females (Tukey's HSD test: $p < 0.01$, 35.61 ± 0.57) and the shortest were the shoots of non-pregnant females (Tukey's HSD test: $p < 0.01$, 29.30 ± 0.88).

In autumn, the controls had longer shoots (32.12 ± 0.86) than the beans treated with urine, no matter if it was urine of pregnant (Tukey's HSD test: $p < 0.01$, 10.20 ± 0.27) or non-pregnant females (Tukey's HSD test: $p < 0.01$, 9.42 ± 0.19). However, the shoot lengths of beans treated with urine of pregnant and non-pregnant females did not differ from each other during autumn and winter ($p > 0.05$).

In winter, the situation was the same as in summer. The longest shoots had controls (16.58 ± 0.56), shorter were shoots of pregnant females (Tukey's HSD test: $p < 0.01$, 6.24 ± 0.15) and the shortest shoots had the beans treated with non-pregnant females (Tukey's HSD test: $p < 0.01$, 37.17, 5.37 ± 0.12).

5.4.3.c Wheat seeds and urine with 1:4 concentration

Significant difference was found in shoot lengths of wheat seeds among three tested groups (control samples, 1:4 diluted urine of pregnant females, and 1:4 diluted urine of non-pregnant females) in summer (one-way ANOVA: $F(2, 674) = 285.28$, $p < 0.01$) and in autumn and winter too (one-way ANOVA: $F(2; 1{,}414) = 204.02$, $p < 0.01$).

The relations among groups were the same in all seasons. The shoot lengths of wheat seeds in controls were always significantly longer (Tukey's HSD test: $p < 0.01$, 44.02 ± 2.82 in summer, 15.48 ± 1.20 in autumn and winter), then the shoot lengths of wheat seeds treated with 1:4 diluted urine. No matter if it was urine of pregnant (Tukey's HSD test: $p < 0.01$, 3.04 ± 0.43 in summer, 2.11 ± 0.14 in autumn and winter) or non-pregnant females (Tukey's HSD test: $p < 0.01$, 1.68 ± 0.17 in summer, 1.77 ± 0.06 in autumn and winter). The shoot lengths of seeds treated with urine of pregnant and non-pregnant females did not differ from each other during all seasons ($p > 0.05$).

5.4.3.d Wheat seeds and urine with 1:14 concentration

In the case of comparing control wheat seeds and wheat seeds treated with 1:14 diluted urine of pregnant and non-pregnant females, the results were very similar to the same analysis with 1:4 diluted urine. Significant differences were found among groups in summer (one-way ANOVA: $F(2; 1,251) = 333.46$, $p < 0.01$) and in autumn and winter too (one-way ANOVA: $F(2; 2,247) = 218.65$, $p < 0.01$).

The shoot lengths of wheat seeds in controls were always significantly longer (Tukey's HSD test: $p < 0.01$, 44.02 ± 2.82 in summer, 15.48 ± 1.20 in autumn and winter), then the shoot lengths of wheat seeds treated with 1:14 diluted urine. No matter if it was urine of pregnant (Tukey's HSD test: $p < 0.01$, 5.90 ± 0.55 in summer, 3.39 ± 0.19 in autumn and winter) or non-pregnant females (Tukey's HSD test: $p < 0.01$, 4.34 ± 0.52 in summer, 3.69 ± 0.14 in autumn and winter). The shoot lengths of seeds treated with urine of pregnant and non-pregnant females did not differ from each other during all seasons ($p > 0.05$).

5.5 Barium chloride test

No relationship was found between the real reproductive status of animals and the results of the barium chloride test. The test was not reliable even if it was assessed for non-pregnancy versus pregnancy (Pearson's chi-squared test: $\chi^2 = 1.61$, $df = 1$, $p = 0.21$), halves of pregnancy (Pearson's chi-squared test: $\chi^2 = 1.62$, $df = 2$, $p = 0.44$), or thirds of pregnancy (Pearson's chi-squared test: $\chi^2 = 1.94$, $df = 3$, $p = 0.59$). The results were similar even if the samples from female Ema were not included in analyses ($p > 0.05$).

No significant difference was found in results of the test from different seasons in non-pregnant females (Pearson's chi-squared test: $\chi^2 = 0.97$, $df = 3$, $p = 0.81$).

In alpacas, the majority (92.86%) of urine samples showed negative reaction. Just 4 positive reactions (7.14%) occurred, 3 in pregnant females and 1 in a non-pregnant female. Because of this, the second hypothesis of this thesis that the urine of pregnant female alpacas mixed together with 1% solution of $BaCl_2$ will not coagulate was rejected.

5.6 Cuboni reaction

No relationship was found between the real reproductive status of alpacas and the results of the Cuboni reaction even if the accuracy was assessed for non-pregnancy versus pregnancy (Pearson's chi-squared test: $\chi^2 = 0.05$, df = 1, p = 0.83), halves of pregnancy (Pearson's chi-squared test: $\chi^2 = 0.09$, df = 2, p = 0.95) and thirds of pregnancy (Pearson's chi-squared test: $\chi^2 = 1.72$, df = 3, p = 0.63). The results were similar even if the samples from female Ema were not included in analyses ($p > 0.05$). Results of the Cuboni reaction in pregnant and non-pregnant alpaca females are provided in Table 14.

No significant difference was found in results of the test from different seasons in non-pregnant females (Pearson's chi-squared test: $\chi^2 = 5.56$, df = 3, p = 0.13).

The third hypothesis of the thesis was that the urine of pregnant female alpacas would show green opalescence after the Cuboni reaction. Green opalescence after the Cuboni reaction was confirmed just in 70.37% of urine samples from pregnant females.

Table 14: Results of the Cuboni reaction in pregnant and non-pregnant females together

Result of the Cuboni reaction	**Reproductive status of females**	
	Non-pregnant	Pregnant
Negative	17.24%	13.79%
Positive	36.21%	32.76%
Number of samples	31	27

6. Discussion

6.1 Sample collection

Problems with collection of urine in some alpaca females had also Volkery et al. (2012). In spite of this, urine samples were always successfully obtained from all alpaca females during this research, what is in contrast with Volkery et al. (2012). The statement of Volkery et al. (2012), that alpaca females could be trained for collection was not confirmed.

The methods for inducing the urination caused a need to urinate in some females. However, urine sampling for pregnancy diagnosis is probably not suitable for large-scale herds of alpacas, because of the relatively long time for obtaining one sample.

6.2 Urinalysis

It was adequate to use diagnostic test strips DekaPhan® Leuco for indicative measurement of pH values. However, these measures were less precise due to less specific pH scale.

Measured pH values are similar to results of other authors. According to Fowler (2011), the urinary pH is alkaline in 94%, neutral in 4%, and acid in 2% of alpacas, and in healthy camelids it should range from 7.00 to 8.50. However, the range of urinary pH of healthy camels was usually between 8.00 and 9.00 (Al-Bashan, 2011).

The range of pH values measured by more precise Duotest® double zone pH-indicator papers was strictly from neutral to slightly alkaline part of the scale. This corresponds with the information about ruminants' urinary pH provided by Sundra et al. (2004). The range of pH values measured by diagnostic test strips DekaPhan® Leuco was wider and corresponds exactly with the statement of Cunningham (1992), who declared that the ruminants' urinary pH values normally varied from 6.00 to 9.00 (Salles et al., 2012). It is known that the pH of urine is influenced by the diet of animals (Sundra et al., 2004; Sullivan et al., 2012), but also by medication or presence of disease (Bartges, 2012). However, the pH of healthy ruminants' urine is alkaline in general (Ryan and Irwin, 2006).

Two females (16.67%) from Alpaca Farm suffered from serious parasitosis and were treated with antibiotics during the research. This could influence the pH values in them (Grosche et al., 2007). However, no differences of pH values were found among

farms. This means that the disease probably did not significantly influence the urinary pH in ill animals, and that the diet of animals on different farms was very similar.

The finding of no difference in pH values of pregnant and non-pregnant alpaca females corresponds with the research of Veena and Narendranath (1993) and Dilrukshi and Perera (2009) in cattle. This means, that the pH could not be considered as a factor that could cause a difference between the results of pregnancy tests in pregnant and non-pregnant alpacas.

The urinary values of specific gravity measured by DekaPhan® Leuco are influenced by the pH values. If the pH values are higher than 6.50 (here in 57 samples, 95%), there can be interference in results (ErbaGroup, 2014). According to Fowler (2011), urinary specific gravity of healthy alpacas and other camelids should range between 1.010 and 1.048. Urinary specific gravity in camels usually ranges between 1.005 and 1.015 (Al-Bashan, 2011).

The higher level of leucocytes in one sample could be influenced by the disease (Grosche et al., 2007). It could be caused also by alkaline pH of the sample (8.50) or its higher bilirubin level (+), because both of these can influence the result of leucocytes measurement according to ErbaGroup (2014).

The detection of nitrites is influenced by diuresis (ErbaGroup, 2014). The positive reaction could be influenced by retention of urine by animals, because the only two positive samples were collected during the first visit on a farm and the animals could be frightened by the presence of an unknown collector.

Urine of healthy alpacas should be negative for protein (Fowler, 2011). The detection of protein in 29 samples (51.79%) could be influenced by urinary pH higher than 8.00 (ErbaGroup, 2014). From other 6 positive samples (10.71%), 4 samples (7.14%) were from the same female, namely Popelka. In Popelka, this could be related to some metabolic problem because this female had also problems with metabolism of zinc. In the remaining 2 samples (3.57%), the pH measured by more precise Duotest® double zone pH-indicator papers was 8.20 and 8.50 so the positive measurement could be caused also by the higher pH level (ErbaGroup, 2014).

Negative glucose results are in agreement with results in healthy alpacas (Fowler, 2011).

Ketones can be detected in urine of healthy alpacas and they are more frequent in urine of pregnant females (Fowler, 2011). However, both positive samples were collected from non-pregnant females.

Bilirubin and higher levels of urobilinogen can occur in urine of healthy alpacas, but the incidence is usually lower (Fowler, 2011). It could be caused by reciprocal influence between levels of bilirubin and levels of urobilinogen during measuring (ErbaGroup, 2014).

The blood is normally not present in urine of healthy alpacas (Fowler, 2011). The positive reaction on blood can occur in samples with extremely high specific gravity (ErbaGroup, 2014). In two positive samples, the value of specific gravity was 1.005. However, these results could be false because the pH values of these two samples were 7.50 and 9.00, and thus could influence the results of specific gravity measurement. The female with the higher level of blood in urine was 20 days after parturition. However, this had probably no influence on the measurement, because the parturition was uncomplicated and the time interval between the parturition and the measurement was relatively long. The females were not mated before the measurements, so this was not the cause too. Both these females were in excellent health status. One probable explanation for the detection of blood in their urine was extremely high real specific gravity of these two samples that remained undiscovered due to high urinary pH values.

6.3 Seed germination test

In general, alpacas' urine inhibited the germination and growth of both kinds of seeds, no matter if it was urine of pregnant or non-pregnant females, in comparison with seeds treated with distilled water. The same results were obtained with the usage of wheat seeds in cattle (Veena and Narendranath, 1993; Krishna Rao and Veena, 2009; Rao Krishna and Veena, 2009; Narayana Swamy et al., 2010), and human (Ghalioungui et al., 1963), and with the usage of mung beans in cattle (Dilrukshi and Perera, 2009; Rao Krishna and Veena, 2009). However, results of Rine et al. (2014) with wheat seeds in cattle were different.

The inhibitory effect of urine on seed germination and growth was higher with the usage of urine with 1:4 concentration than with the urine with 1:14 concentration. These results correspond with the findings of Dilrukshi and Perera (2009), who used mung beans in cattle.

Usually, seeds germinated and grew better in the urine of pregnant females than in the urine of non-pregnant females. The same results were obtained with human urine of pregnant and non-pregnant females (Hoffmann, 1934; Ghalioungui et al., 1963). However, it was conversely in cattle (Veena and Narendranath, 1993; Dilrukshi and Perera, 2009; Krishna Rao and Veena, 2009; Rao Krishna and Veena, 2009; Narayana Swamy et al., 2010), mithuns (Perumal, 2014), buffaloes, sheep, and goats (Rao Krishna and Veena, 2009).

It has been shown that mung beans treated with urine with 1:14 concentration and wheat seeds treated with urine with 1:4 and 1:14 concentration are not suitable for the seed germination rates counting as a pregnancy diagnostic test in alpacas. Mung beans treated with urine with 1:4 concentration could be usable for pregnancy diagnosis by seed germination rates counting. For shoot lengths measuring as the pregnancy diagnostic method in alpacas both mung beans and wheat seeds could be used, but in case of wheat seeds just the urine with 1:4 concentration was usable.

Season seemed to be an important factor, which can influence the results of the seed germination test. However, it was not considered by any other author. The influence of the season is probably caused by different duration of the daylight or by temperature fluctuations in different seasons.

Some facts should be considered in connection with findings of this research. There is a possibility that some metabolite of P4 could be the factor that causes differences between pregnant and non-pregnant females (Rao Krishna and Veena, 2009), and it should be taken into consideration, because the influence of the female with persisting CL was not considered in these analyses. Further, in these experiments, the effect of *post partum* period was not considered, as it was in the cattle (Veena and Narendranath, 1993). The late establishment of the experiments from four collections could have some impact on the results. However, there are also authors, who did not specify the storage time of urine before the establishment of experiments at all, e.g. Veena and Narendranath (1993), or Dilrukshi and Perera (2009).There is also a problem with the subjectivity of considering which seed had already geminated and which had not.

Further research should be focused on verification of these findings and specification of the reliability of this test in alpacas. For other trials with the seed germination test in alpacas, usage of mung beans as the only kind of seeds could be recommended. Also, the change of urine and seeds colour (Krishna Rao and Veena, 2009;

Rao Krishna and Veena, 2009) was observed but not systematically. It could be included in the experimental design of further research.

6.4 Barium chloride test

The finding that the barium chloride test is not suitable for pregnancy diagnosis in alpacas corresponds with the results of Krishna Rao and Veena (2009) in cattle, and Haberová et al. (2011) in Bactrian camels. However, they are in contrast with successful experiments in pigs (Ndu et al., 2000a; Ndu et al., 2000b; Lalrintluanga and Dutta, 2009), and Arabian camels (Banerjee, 1974; Purohit, 2010).

The urine of female alpacas usually coagulated after the addition of 1% $BaCl_2$ solution. Similar results were obtained by Ohazurike (1990) in non-pregnant ewes and goats. However, 2% $BaCl_2$ solution was used in them and maybe the higher concentration caused the precipitation in all samples (Ndu et al., 2000a; Ndu et al., 2000b).

What causes the precipitation of alpacas' urine is not sure. The short-term presence of faeces in urine sample during the collection was rejected as a factor that could influence the result of the reaction. It is because the precipitation occurred also in samples that were collected without any contamination by faeces.

Increased number of crystals was observed in alpacas' urine samples after refrigeration, which was described by Albasan et al. (2003) in the urine of cats and dogs. These crystals could be the factor, which caused the precipitation of almost all urine samples in alpacas, because the urine was not filtrated before the barium chloride test processing. However, filtration of urine before the barium chloride test processing was not described by any author that was cited in this thesis.

Other factors that were suggested by other authors and described in the chapter 2.3.4 Barium chloride test should be considered too. However, it was concluded that the barium chloride test is not suitable pregnancy diagnostic test for alpacas.

6.5 Cuboni reaction

Similar results with many false positive and false negative reactions were obtained by Heller (1940) in pigs, and also by O'Moore (1947) in cattle. False negative reactions occurred in alpacas probably because the Cuboni reaction is based on reactions of free oestrogens in urine (England, 2008), and the urinary oestrogen level is highly increased just during the first and last month of pregnancy in alpacas (Bravo et al., 1996; Volkery et

al., 2012). However, false positive reactions occurred in non-pregnant females. This could be caused by higher oestrogen levels in the time of heat in alpaca females, which was considered by Haberová et al. (2011) in connection with false positive Cuboni reactions in Bactrian camels.

The results are in contrast with findings of other authors, who confirmed the reliability of the test for some parts of pregnancy in horses (Cuboni, 1934; Weber, 1937), ponies (Cox and Galina, 1970) and Bactrian camels (Fedorova et al., 2013).

In relation to alpacas, camels are the most related animals on that the Cuboni reaction was tested. In spite of this, in alpacas the Cuboni reaction was not successful in pregnancy diagnosis, although it was successful in Bactrian camels in the 3rd third of pregnancy (Fedorova et al., 2013). However, the failure of this method in alpacas could not be caused by a different way of processing of samples or different evaluation of the results. It is because the urine samples of Bactrian camels collected by Fedorova et al. (2013) were subjected to the Cuboni reaction also in the SVI Prague.

It was concluded that the Cuboni reaction is not suitable for pregnancy diagnosis in alpacas at all.

7. Conclusions

It has been shown that non-invasive urine sampling for pregnancy diagnosis in alpacas is possible, but this method is suitable probably just for smaller herds.

In case of the seed germination test, it was found out that the urine of alpacas usually inhibited the germination and growth of seeds in comparison with distilled water. The seeds germinated and grew always better in the urine with 1:14 concentration than in the urine with 1:4 concentration, no matter if it was urine of pregnant or non-pregnant females. Usually, seeds germinated and grew better in the urine of pregnant females than in the urine of non-pregnant females, which disproved the first hypothesis of this thesis. Mung beans seemed to be more suitable for the seed germination test in alpacas. Further research should be focused on verification of these findings and specification of the reliability of this test in alpacas.

The second hypothesis of the thesis was rejected, because the urine of pregnant female alpacas mixed together with 1% solution of $BaCl_2$ almost always coagulated. The third hypothesis could be confirmed just in 70.37% of cases, because the urine of pregnant female alpacas did not always show the green opalescence after the Cuboni reaction. The barium chloride test and the Cuboni reaction were concluded as not suitable methods of pregnancy diagnosis in alpacas because their results did not correspond with the reproductive status of animals.

There is a lack of pregnancy diagnostic methods, which could be accomplished just by breeders themselves. Still, observing the reaction of a female on the presence of a male is the most reliable and early applicable non-invasive pregnancy diagnostic method in alpacas, which can be practised by common breeders. This method cannot be used by the breeders that breed just female alpacas and do not own male individuals. However, this method is usually considered by Czech breeders as a supplementary method to decide whether the female should be rebred. More often, ultrasound and P4 blood level tests are used. However, performance of these methods requires a veterinarian or a well experienced sonographer in case of ultrasound diagnostics. Invasive character of P4 blood level tests is disadvantageous too. Because of this, searching of new simple non-invasive methods for pregnancy diagnosis in alpacas is important.

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Annexes

List of Annexes:

Annexe 1

The world list of ten most bred domestic animals in 2011:

Order	Animal	Number of heads
1.	Chickens	20,708,002,000
2.	Cattle	1,426,389,031
3.	Ducks	1,323,854,000
4.	Sheep	1,093,566,764
5.	Pigs	967,164,630
6.	Goats	924,145,893
7.	Rabbits and hares	894,955,000
8.	Turkeys	467,685,000
9.	Geese and guinea fowls	381,213,000
10.	Buffaloes	195,397,515

Source: FAO (2012)

Annexe 2

Photos of female alpacas, which were included in the research:

All females were photographed individually or with their calves, if they gave birth during the research or shortly before its start. Exceptions are the females from Centre of active rest – Riding Club Bitozeves o.s. of Mrs. Pavlína Kňavová, that were photographed as a group; Karlottka and Blanka, whose calves died few days after parturition.

Centre of active rest – Riding Club Bitozeves o.s. of Mrs. Pavlína Kňavová:

Group of four alpaca females on which the collecting of urine was tested (photo: author)

Alpaca Farm of Mr. Radek Vaněk:

Kumasi (photo: author)

Minea and her calf (photo: author)

Morena and her calf (photo: author)

Tonja and her calf (photo: author)

Alpakafarma.cz of Mrs. Eleonora Hlaváčová:

Aranka (photo: author)

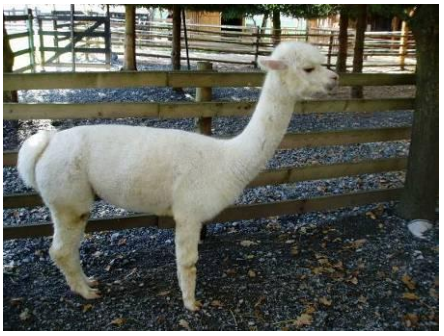
Ema (photo: author)

Kapička (photo: author)

Popelka and her calf (photo: author)

Farm of Mrs. Jana Dufková:

Alina (photo: author)



Blanka, whose calf died (photo: author)

Karlottka, whose calf died (photo: author)

Rozárka and her calf (photo: author)

Annexe 3

The composition and recommended dosage of compound feed for alpacas from the manufacture Výroba krmných směsí - Sehnoutek a synové v.o.s.:

Composition:

- alfalfa, oat, corn
- sunflower oil meal, flax seed, soya oil meal
- $CaCO_3$, NaCl, $Ca(H_2PO_4)_2 \cdot H_2O$, $CuSO_4 \cdot 5H_2O$
- vitamin A, vitamin D3, vitamin E

Qualitative parameters:

- moisture: 14.0%
- crude protein: 13.7%
- fibre: 16.3%
- ash: 5.0%
- vitamin A: 40,000 IU/kg
- vitamin E: 404 mg/kg
- vitamin D3: 10,000 IU/kg
- Cu: 36 mg/kg

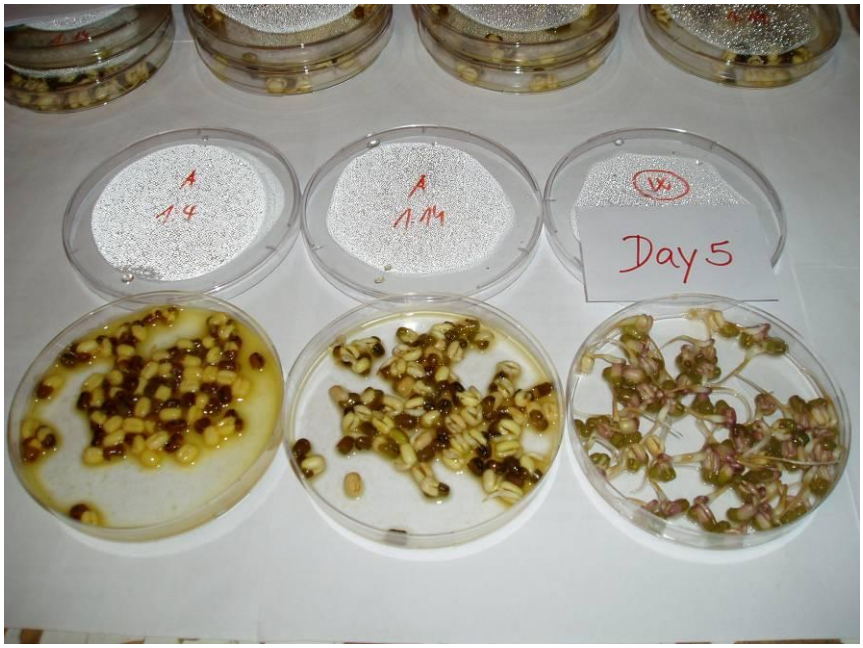
Dosage:

- basic dosage for alpacas: 300 g per 50 kg of live weight
- pregnant female alpacas: 350-450 g per 50 kg of live weight

Source: Hlaváčová (2010)

Annexe 4

Seed germination test with mung beans and winter wheat seeds after 5 days:



Comparison of mung seeds, which were in the urine with concentration 1:4, in the urine with concontration 1:14 and in distilled water for 5 days, respectively from the left (photo: author)

Comparison of winter wheat seeds, which were in the urine with concentration 1:4, in the urine with concontration 1:14 and in distilled water for 5 days, respectively from the left (photo: author)

Annexe 5

Cuboni reaction processing by Bc. Karin Čadová in the SVI Prague:

Urine filtration (photo: author)

Adding of HCl (photo: author)

Solution in the boiling water bath (photo: author)

Separating of the toluene layer (photo: author)

Water bath with 80 °C (photo: author)

Results of the Cuboni reaction (photo: author)

Annexe 6

Example of protocol with results of the Cuboni reaction from the SVI Prague:

Státní veterinární ústav Praha

oddělení chemie

165 03 Praha 6-Lysolaje, Sídlištní 24; tel.+420 251031700; fax.+420 251031335; e-mail: chemie@svupraha.cz

Protokol o zkoušce

Číslo vzorku : 11273-11280/13
Zakázka : 5614/13
Odesílatel : Kubátová Anna, Praha
Plátce : Kubátová Anna, Praha
Analýza provedena ve dnech :

Datum doručení : 28.11.2013
Datum vyřízení : 16.12.2013

28.11.2013 - 16.12.2013

Č.vzorku:	Popis vzorku:
11273	moč lama č. 1
11274	moč lama č. 2
11275	moč lama č. 3
11276	moč lama č. 4
11277	moč lama č. 5
11278	moč lama č. 6
11279	moč lama č. 7
11280	moč lama č. 8

Výsledky vyšetření:

Číslo vzorku:	11273	11274	11275	11276	11277
březost	negat.	pozit.	pozit.	pozit.	negat.

Číslo vzorku:	11278	11279	11280
březost	negat.	pozit.	pozit.

Použité metody:
březost - Cuboniho reakce

Výsledky jsou bez záruky, metoda je určena pro stanovení březosti klisen.

Poučení : protokol může být reprodukován jedině celý, jeho části pouze se souhlasem SVÚ Praha. Výsledky zkoušek se týkají pouze vzorku uvedených v protokolu.

STÁTNÍ VETERINÁRNÍ ÚSTAV Praha

Ing. Jan Rosmus
vedoucí oddělení chemie

Rozdělovník :
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