CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Faculty of Tropical AgriSciences

Genetic Monitoring and Reproduction Management of Captive Ungulate Populations

DISSERTATION THESIS

Prague 2020

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Declaration

Hereby the author of the thesis declares that this thesis titled "Genetic Monitoring and Reproduction Management of Captive Ungulate Populations" includes original text that has been written independently by the author except for the section Results where all the co-authors of included articles were declared and all of them agreed that the articles will be published as a part of this thesis. Moreover, the author declares that all sources the author used have been quoted and acknowledged by means of the complete reference list in the section References according to the Citation rules of the FTA at the end of the thesis (except the sources used in individual articles that have been quoted and acknowledged according to the style of the journal where they have been published and they were listed separately within each article).

The author states that the work has not been and is not being submitted for any other degree to this or any other university.

In Prague on July 16, 2020

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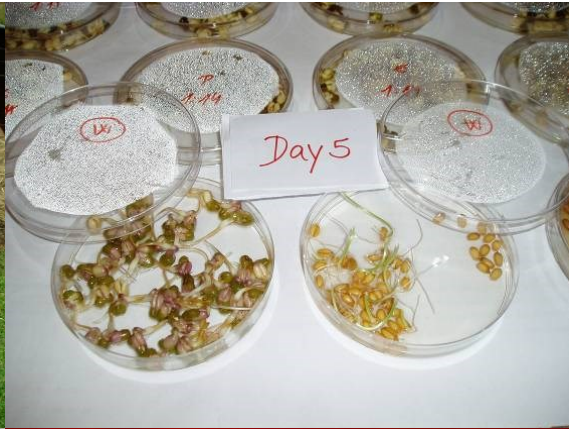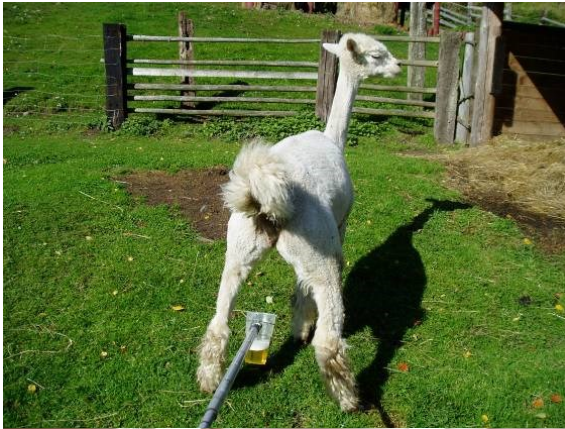Anna Kubátová

Acknowledgements

Photo by author (upper four pictures) and Derbianus Conservation, z. s. (lower four pictures).

Abstract

Population management is an important tool for captive breeding programs and its methods may differ according to the species and its population characteristics. In the case of endangered species suffering from small population sizes, the main goal is always conservation of the species over time. This means an increase in the number of individuals and maintenance of an adequate gene pool to preserve sufficient variability and reduce the probability of extinction. In captive and semi-captive populations, attainment of this goal is closely connected with the methods of genetic monitoring and reproduction management that are essential for achieving healthy and viable populations in a long-term period. This dissertation thesis has been directly focused on genetic monitoring in critically endangered Western Derby eland (*Taurotragus derbianus derbianus*) and reproduction management in various domestic ungulates living in tropics – alpacas (*Vicugna pacos*), Bactrian camels (*Camelus bactrianus*), cattle (*Bos taurus*) and donkeys (*Equus asinus*). They can be used as model organisms for their related species under local or overall threats, e.g. antelopes and other bovids, vicuñas, wild Bactrian camels, African wild asses, Grevy's zebras or Przewalski's horses. Specifically, the impact of different population managements applied in two semi-captive populations of *Taurotragus* spp. via the comparison of their population structure characteristics, detection of possible interspecific hybridization within the genus *Taurotragus* by microsatellite markers and non-invasive methods of pregnancy diagnosis from urine (barium chloride test, Cuboni reaction, seed germination test) in alpacas, Bactrian camels, heifers, and donkeys, has been explored. The results of this study could be used for proper population management in conservation programmes focused on examined endangered taxa or ungulates related to our model species. And last but not least, discovered facts should contribute to domestic animal husbandry of selected tropical species.

Keywords: *Bos taurus*; *Camelus bactrianus*; *Equus asinus*; *Taurotragus* spp.; *Vicugna pacos*

Contents

1　Introduction

Ungulates (Ungulata) are a group of "hoofed" animals including species of two orders, odd-toed ungulates (Perissodactyla) and even-toed ungulates (Artiodactyla, Cetartiodactyla without Cetacea) (Waddell et al. 2001; Gippoliti et al. 2018). Members of this superorder have different conservation statuses (IUCN 2017), they are bred under different conditions and for different purposes. They represent important domestic animals, game species, hobby animals, and pets and many of them are also bred in zoos (Long 2003).

Ungulates are one of the most important groups of domestic animals bred mainly for production purposes. Food and Agriculture Organization of the United Nations recognizes 17 types of most bred domestic animals (except bees) and ten of them are ungulates both even-toed (cattle, sheep, goats, pigs, buffaloes, camels, other camelids) and odd-toed (horses, asses, mules). In the year 2018, five of them were placed on the world list of the ten most bred domestic animals together with variable species of poultry, rabbits, and hares (FAO 2018). However, despite the fact that domestic ungulates are abundant on the species level, endangered breeds facing extinction can be recognized (Meuwissen 2009). Many of the domestic ungulates are also bred as pets. Most common companion animals from the ungulate group are equids (WHW & EG4A 2015; APPA 2018), but other species of ungulates are becoming more and more popular as hobby animals, e.g. llamas, alpacas (Smith et al. 1994) or miniature pigs (Van Metre & Angelos 1999).

Nevertheless, domestic ungulates are not the only species bred for production purposes. Although the most utilized game species vary among geographic areas (Gortazar et al. 2006), the worldwide majority of meat-producing large game species are ungulates (McCormick 2003). Except for game farms and ranches, where animals are bred mainly for harvesting, endangered wild ungulate species are frequently bred for conservation purposes, e.g. in reserves or zoos (Gippoliti et al. 2018). According to the IUCN (2017), 42.6 % of species of even-toed ungulates and even 81.3 % of species of odd-toed ungulates are now threatened or already extinct.

Population management is important in all these types of captive breeding and its tools vary according to the population goals. The goals depend on population

characteristics, mainly on its size (small × desired × abundant) and breeding purpose (conservation × production/harvest) (Williams et al. 2002), that are usually also connected to the conservation status of the species (EAZA 2012) or the breed in the case of domestic animals (Meuwissen 2009).

Conservation via captive breeding can be realized both *in situ* and *ex situ*, and population management may differ between these two types (Coonan et al. 2010). Nevertheless, the One Plan Approach to wildlife species conservation is preferred nowadays. It is an integrated approach to species conservation consisting of management strategies and conservation actions by all responsible parties for all populations of a species, whether inside or outside their natural range (CPSG 2018). A similar approach should also be applied in the case of endangered breeds and thus FAO established the Global Plan of Action for Animal Genetic Resources (Hoffmann et al. 2011).

2   The aims of the thesis

The main aims of the thesis were:

1) Verify the possibility to use basic urinalysis as a simple pregnancy diagnostic method in alpacas (*Vicugna pacos*) and Bactrian camels (*Camelus bactrianus*).

2) Verify the applicability of three non-invasive pregnancy diagnostic tests from urine, the barium chloride test, the Cuboni reaction and the seed germination test, in alpacas (*Vicugna pacos*), donkeys (*Equus asinus*) and heifers (*Bos taurus*).

3) Test for the presence of interspecific hybrids between semi-captive Western Derby elands (*Taurotragus derbianus derbianus*) and Cape elands (*Taurotragus oryx oryx*) living in a multispecies enclosure in Bandia Reserve, Senegal.

4) Compare the population genetic parameters of the two divergently managed species and evaluate the effect of the applied population management in the populations of Western Derby elands (*Taurotragus derbianus derbianus*) and Cape elands (*Taurotragus oryx oryx*) living in a multispecies enclosure in Bandia Reserve, Senegal.

3   Literature review

The most visible result of applied population management is usually the impact of reproduction management on the number of individuals that are bred (Williams et al. 2002). However, the genetic status of populations should be also considered because the maintenance of a healthy and self-sustaining population should always be a priority in all types of captive breeding (WAZA 2005; EAZA 2012; Penfold et al. 2014). These characteristics are not important just for the long-term survival of the population, but also for its possible sustainable production performance (Berry et al. 2011).

As was stated in the Introduction, there are different approaches to populations with different goals. So-called open or closed herd operations can be applied in domestic animal production when the increase in stock is desired (Hallewell et al. 2016). The animals or their gametes can be sourced either elsewhere (in another institution or in the wild) (EAZA 2012) or the reproduction of the particular population can be supported, e.g. by the competitiveness of the males, hand-rearing, supplementary feeding, etc. (Burch et al. 1995; Mysterud et al. 2004; Mbatha & Bakare 2018).

In case of the need to reduce the population size, individuals can be transferred to other captive breeding facilities (sold, exchanged for other species) or to the wild (conservation translocations), their breeding can be suspended or delayed (e.g. by male and female separation, castration, contraception) or surplus individuals may be culled/harvested (van der Waal & Dekker 2000; Raphael et al. 2003; WAZA 2005; Borkowski et al. 2009; EAZA 2012; Bowyer et al. 2014; Penfold et al. 2014).

Populations of the desired abundance are managed only to maintain their population size (Williams et al. 2002) which includes also switching of breeding individuals between institutions. These exchanges of breeding individuals can benefit the captive populations by increasing genetic variability and effective population size while reducing inbreeding and divergence from wild populations (Norton & Ashley 2004). Other options include methods like "breed and place" or "breed and cull" (Penfold et al. 2014).

Today, there are many tools that can help to evaluate the state in which the population currently is, and based on this to decide the next steps of the population management. The most common electronic system that enables the facilitation of such decision making for zoos nowadays is called Species360 ZIMS, i.e. Zoological Information Management

System (Species360 2018). Nevertheless, there are many other software tools including unique programmes for the management of both domestic animals (Magne et al. 2010; Corner-Thomas et al. 2016) and game species (NGMD 1996; GMS 2018).

However, special attention is paid to small populations and their management that must deal with obstacles and difficulties related to small population sizes. Even that this issue concerns also domestic animals (e.g. endangered breeds facing extinction) (Meuwissen 2009), the main topic in which the question of small populations is discussed are conservation programmes for wildlife.

3.1 Small Populations

The reasons which are responsible for small population sizes differ. In the wild it can be a population bottleneck caused by a sudden and significant drop in livestock numbers, e.g. due to poaching or overhunting of either the species or its prey, incidence of disease, or natural disaster (Primack 2000). However, there are also species that have naturally lower abundance as endemic species with limited geographic range (Hobbs et al. 2011). Concerning domestic animal breeds, the numbers of individuals in the population usually decrease when one breed is replaced by another more productive (Meuwissen 2009). Due to small sizes, these populations can be more vulnerable to genetic problems and demographic and environmental fluctuations (Primack 2000).

Genetic variability of populations represents an essential pillar that enables survival through the possibility of adaptations in changing environments. In small populations with limited gene pool, the impact of genetic drift and inbreeding, which can contribute to the reduction of genetic variability and fixation of harmful alleles, is increased (Primack 2000; Courchamp et al. 2008). On the other hand, as the frequency and homozygosity of recessive deleterious alleles increase, selection can remove (purge) them from a population and thus reduce the genetic load (Pekkala et al. 2012). In the case of domestic animals, inbreeding also plays a role in breeds creation as a process that enables allelic fixation when a selection for a concrete trait of economic importance is carried out (Kristensen & Sorensen 2005).

Even populations that are relatively abundant may face problems connected to reducing genetic variability and become extinct because of the possible existence of small isolated sub-populations with no gene flow. Similar problems are typical for captive populations which size is limited by space, and the individuals are scattered

among institutions worldwide so the gene flow is reduced. These populations, especially of endangered species, often come from a small number of founders, and from the genetic point of view there is a strong influence of the founder effect (Primack 2000; Frankham et al. 2002; Frankham 2008).

To eliminate negative influences of mentioned phenomena in conservation programs as much as possible, it is necessary to apply appropriate population management that will ensure the increase of population size on the desired level and reduce the probability of its extinction via keeping sufficient genetic variability and demographic balance. In captive and semi-captive populations attainment of this goal is closely connected with the methods of genetic monitoring and reproduction management which are always essential for achieving healthy and viable populations in a long-term period (Lande & Barrowclough 1987; Williams et al. 2002).

3.2 Reproduction Management

Reproduction management is an essential part of captive breeding, which corresponds to the fact that connected research creates an important ratio of the studies that are conducted in zoos worldwide. The analysis of papers on mammals in the 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 represented 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 published from 2000 to 2011 regarding research of zoo ungulates, reproduction research was in the second place after the 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 both livestock (Gargiulo et al. 2012) and wildlife (Monfort et al. 1993).

For successful natural breeding healthy and fertile breeding pairs are necessary, therefore fertility focused examinations are quite common in captivity (Penfold et al. 2005; Metrione & Harder 2011). Conservation programmes including just one founding individual of a certain sex can be very risky especially when the fertility of the specimen is unknown (Pinto 2009; Zemanová et al. 2015). However, even the presence of fertile breeding pairs with no health problems does not guarantee a successful mating, conception, pregnancy, and finally parturition. Sometimes the pairs are just behaviourally

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or sexually incompatible (Metrione et al. 2014; Schulte-Hostedde & Mastromonaco 2015), but also other behaviourally caused reproductive failures can occur (Lindburg & Fitchsnyder 1994), e.g. deficiency in sexual motivation in captive males (Zhang et al. 2004) due to various reasons including lack of rivals (Bian et al. 2013).

To solve these problems it is possible to make changes in breeding pairs, change the system of natural breeding in connection to housing and enclosures, or just to simulate it (Bian et al. 2013). However, in case of unsuccessful natural breeding, artificial breeding methods still offer a chance for successful breeding. Reproductive biotechnologies like artificial insemination, embryo transfer (Fernández-Baca 1993; Vaughan 2004; Penfold et al. 2005), *in vitro* fertilization (Sumar 1999), ovarian super-stimulation, nuclear transfer (Miragaya et al. 2006), follicular synchronization, oocyte maturation or embryo cryopreservation (Adams 2007) were already used in ungulates, but their usage varies both across species and breeding facilities. In the case of domestic animals, these procedures can allow the propagation of genetically superior production animals (Miragaya et al. 2006), and in zoos they can help to maintain genetic diversity among other effects (Ballou 1984).

Nevertheless, for the application of the majority of the aforementioned biotechnologies in animal reproduction, knowledge of both the domestic and wildlife female reproductive cycles and their oestrous periods is necessary (Lasley & Kirkpatrick 1991; Heersche & Nebel 1993; Alagendran et al. 2007; Kusuda et al. 2007). Without proper oestrous detection, reproduction of a significant ratio of domestic ungulates nowadays dependent on artificial insemination would not be possible. Moreover, detection of oestrus can play an important role even in natural breeding of species in which signs of pro-oestrous or oestrus are not obvious, and in which proper timing of joining separately kept males and females for the purpose of mating is crucial. Proper oestrus detection also highly increases the probability of conception (Caro & Laurenson 1994; Knott et al. 2010) and can be a key solution in the case of former infertility of the breeding pair.

Pregnancy diagnosis is also essential for better reproductive management in animals. In domestic animals, reproductive efficiency represents an important criterion for production (Dilrukshi & Perera 2009; Balhara et al. 2013), which leads to profitable animal husbandry. Early pregnancy diagnostics enables to detect females which did not conceive and should be rebred, treated (Balhara et al. 2013) or weeded out from

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reproduction, and subsequently usually culled (Lalrintluanga & Dutta 2009; Bah et al. 2010; Holendová & Čechová 2010; Perumal 2014) in the shortest interval possible to prevent the losses.

However, pregnancy diagnosis can be important also in wildlife (Bashaw et al. 2010) or zoo animals (Kleiman et al. 2010). It enables to gather 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 & Fricke 2005), or to estimate the date of birth and be prepared for it (Kleiman et al. 2010). It also allows the detection of the number of embryos/foetuses, to recognize their sex (Lamb & Fricke 2005), and monitor their development (Suguna et al. 2008; Lueders et al. 2009). Early pregnancy diagnosis also enables to change the nutrition of the females according to the pregnancy status (Parker et al. 2009), more intensively monitor females with some disorders (Kleiman et al. 2010), or timely separate males and females when appropriate (Metrione et al. 2014).

Reproduction management is connected not only with the methods that facilitate the reproduction, but also with methods that can restrict it, as was mentioned at the beginning of the literature review. In captive breeding facilities, these methods are used usually when the population exceeds the capacity of animal housing, and their export is not somehow possible. All the methods have their pros and cons and their selection must be carefully considered (WAZA 2005; EAZA 2012). However, methods restricting reproduction are used even in small populations where the reproduction is generally desirable. In these cases, they are applied to individuals with over-represented genes in the population (Shen et al. 2009).

3.3 Genetic Management

To apply proper genetic management in small captive populations, it is necessary to know basic information about the kinship and genetic variability across the individuals (Thévenon & Couvet 2002) that are usually recorded in studbooks. Studbooks of domestic animals have a long tradition and thus the availability of data is mostly sufficient (Glatston 1986). Although nowadays studbooks for wildlife exist (e.g. European studbooks (ESBs) within European Association of Zoos and Aquaria (EAZA 2012) or International studbooks (ISBs) under the auspices of World Association of Zoos and Aquariums (WAZA 2005)), knowledge of kinship across these populations

is often limited. It reaches only 20-25 % in some ungulate species kept in European zoos, and some of the populations are not monitored at all, e.g. nilgau (*Boselaphus tragocamelus*) or gerenuk (*Litocranius walleri*) (Simonsen 2015).

However, breeding management in captivity is usually highly influenced by the conservation status of the species, and thus there are also species with better information background. These are mainly endangered species for which special *ex situ* conservation programs are created, e.g. European Endangered Species Programmes (EEPs) of EAZA in Europe (EAZA 2012). Nevertheless, even the studbooks do not guarantee reliable information concerning the genetic status of animals. It is because they are based on the assumption that founders are unrelated and non-inbred which is not necessarily true. Only proper genetic monitoring including genetic analyses can reveal the real situation of the populations (Witzenberger & Hochkirch 2011).

The genetic background of the population should be always considered from the beginning, i.e. since the establishment of the conservation programme. In optimal circumstances, there are more suitable candidate populations in the wild (in other breeding facilities when considering endangered breeds) from which intended founders of the backup population can be selected. All founders should be ideally unrelated and their number sufficient to establish more breeding pairs to avoid kinship already in the first generation (Pinto 2009; Witzenberger & Hochkirch 2011; Zemanová et al. 2015). However, this is not always possible in case of critically endangered species or breeds in which animal numbers have decreased to such an extent that backup breeding programme must be established with just a few remaining founders (Roldan et al. 1998). As an example of an extreme situation in wildlife can serve e.g. the final attempt to save the subspecies of northern white rhinoceros (*Ceratotherium simum cottoni*) via hybridization with the conspecific southern white rhinoceros (*Ceratotherium simum simum*) despite their natural long time period of geographic and thus genetic isolation (Harley et al. 2016). Similarly, within the conservation programme for the Czech Red cattle, insemination doses of the Polish Red cattle were used due to the limited gene pool of the Czech national breed (Majzlík 2015). Nevertheless, there should be always an effort to avoid the possibility of both inbreeding and outbreeding depression if possible (Witzenberger & Hochkirch 2011).

Captive and semi-captive populations can also face problems with interspecific hybridization. At the present time, the process of interspecific hybridization is considered

a very important factor that endangers biodiversity and the existence of many species (Perry et al. 2002). Although the hybridization occurs in nature, anthropogenic hybridization caused by human interference is quite common and it may lead to breeding of previously reproductively isolated taxa. It happens usually in related taxa which were initially geographically isolated and then artificially joined, e.g. in intentional breeding programs, accidentally in the reserves or ranches, or by mistake in zoos when the species are incorrectly determined, or due to mixed-species exhibits that are more and more popular because of higher attractiveness for visitors (Benirschke 1967; Hosey et al. 2013; Dalton et al. 2014). Besides, anthropogenic interspecific hybridization occurs also between domestic species and their wild ancestors which can happen either by mistake as in spontaneous hybrids of gaur and cattle (Bongso et al. 1988; Nijman et al. 2003), or other ungulates (Randi 2008), or on purpose due to mainly commercial purposes, which is the case of e.g. pacovicuña, i.e. alpaca/vicuña hybrid (Lichtenstein et al. 2008), or American bison population historically introgressed by cattle (Hedrick 2009).

Common post-zygotic reproductive isolation mechanism is hybrid sterility which appears in the early stages of speciation of various organisms (Storchová et al. 2004; Pinto et al. 2016). However, even in the absence of introgression, hybridization of species in the wild is undesirable due to wasted reproductive effort (Allendorf et al. 2001), and a reduction in productivity (Dalton et al. 2014). Such hybridizations can jeopardize whole conservation programmes by this way and thus there is a high need for its monitoring as well as genetic variability assessment.

3.4 Invasive and non-invasive research methods

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 (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 animals. In some types of research, where differentiation of individual animals is not required, non-invasive methods provide data even without observing the animals (Waits & Paetkau 2005). Because of all these positives, non-invasive methods are more and more popular nowadays (Garshelis 2006; Kleiman et al. 2010).

Every handling or closer examination of bigger non-tamed animals, e.g. in zoos or reserves, requires physical or chemical restraint which can be dangerous for both the animal and personnel, and it also very stressful for the animals (Kleiman et al. 2010). In view of this fact, common invasive and non-invasive diagnostic methods such as blood collections (Volkery et al. 2012) or ultrasonography (Rees 2011) are not always applicable in these animals, and the keeper can only estimate the status of the animal according to his/her experience or observations which are usually not much reliable (Muhammad et al. 2000). Therefore, there is a need of new simple, quick, economical and reliable methods applicable in such animals.

More detailed introductions and literature reviews of individual research topics are described in particular articles included in section 5 – Results (p. 15).

4   Material and Methods

4.1 Animals

Concerning reproduction monitoring, female alpacas (*Vicugna pacos*), cattle heifers (*Bos taurus*) and jennies (*Equus asinus*) from six Czech private farms (Liska, Veska, Zamosti-Blata, Ounuz, Kostelec nad Vltavou, Vendoli) and Bactrian camels (*Camelus bactrianus*) from four Czech and Slovakian zoos (Pilsen, Prague, Usti nad Labem, Bratislava) were included in the project.

Regarding genetic monitoring, two eland species from the tribe Tragelaphini were included in the research. The first species was represented by native Senegalese individuals of the western subspecies of Derby eland (*T. derbianus derbianus*) from semi-captive populations in Bandia and Fathala reserves in Senegal, Western Africa. The second species was represented by individuals of the common eland (*T. oryx*), specifically the subspecies cape eland (*T. oryx oryx*), that were introduced to Bandia Reserve from South Africa.

4.2 Sample collections

Data from urine collections in female alpacas (n = 60), female camels (n = 62), heifers (n = 86), and jennies (n = 54) that were carried out from September 2010 to December 2014, were already available. Urine was sampled repeatedly in 2 to 9-week intervals in non-pregnant females as well as in pregnant females to cover different stages of pregnancy. Urine was collected during spontaneous urination of animals into plastic cups held in hand or fastened to a long rod (Fedorova et al. 2015).

Blood, hair, and tissue samples collected from the Western Derby elands and cape elands in the period 2005-2015 were already available for analyses thanks to the cooperation with the NGO Derbianus Conservation, Bandia and Fathala reserves, and Directorate of National Parks of Senegal. Additional blood and tissue samples were collected in 2016 and 2017 again in cooperation with the mentioned organizations. In total, 143 samples of Western Derby elands (including 16 samples from a total of 26 potential hybrids that were born until June 2017) and 41 samples of cape elands were finally available for analyses. Blood and hair samples were collected from immobilized animals during their transport between two breeding herds of the same

reserve, or between two breeding herds of different reserves (Koláčková et al. 2011). Tissue samples were obtained directly from animal carcasses or from living individuals by biopsy darts.

4.3 Sample processing

Urinalyses have been carried out immediately after the collection of urine via Duotest® double zone pH-indicator papers and DekaPhan® Leuco diagnostic test strips. Seed germination tests and barium chloride tests were done directly in the laboratory of the Department of Animal Science and Food Processing, Faculty of Tropical AgriSciences (FTA), Czech University of Life Sciences Prague (CZU). Seed germination tests were processed with the usage of mung beans (*Vigna radiata*) and wheat seeds (*Triticum spelta*) (Rao Krishna & Veena 2009). For barium chloride tests, 1% barium chloride was used (Ndu et al. 2000b). Cuboni reactions were carried out by the State Veterinary Institute Prague.

The majority of genetic analyses were carried out in the laboratory of molecular genetics of the FTA, CZU. Here, DNA isolations, DNA concentration measurements, PCRs, and electrophoreses were done. For fragmentation analyses, processed samples were transferred to the specialized laboratory of the Faculty of Environmental Sciences, CZU. QIAGEN® DNeasy® Blood & Tissue Kit and Geneaid™ Genomic DNA Mini Kit (Blood/Cultured Cell) were used for DNA extractions. For genetic monitoring and evaluation of interspecific hybridization, 12 fluorescently labelled microsatellite primers were used (Zemanová et al. 2015; Štochlová 2016).

4.4 Data analyses

The results of non-invasive methods of pregnancy monitoring using urine were compared with the current reproductive statuses of animals at the moment of sampling with the usage of StatSoft CR StatisticaCz 12 program.

Allele lengths of individual loci were scored manually in GeneMarker® Version 2.2.0, SoftGenetics LLC® (Matschiner & Salzburger 2009), and then binned by AutoBin (Castagneyrol et al. 2012). The newly gained input data were analysed in other population genetics programs, e.g. Structure 2.3.4 (Yang et al. 2005), GenAlEx 6.502 (Peakall & Smouse 2012), Genetix 405 (Belkhir et al. 1996-2004), GenePop 4.2 (Rousset 2008),

FSTAT 2.9.3.2 (Goudet 1995), and thus population structure, inbreeding coefficient, fixation index, and other characteristics were determined.

Materials and methods are described in detail within each specific article in section 5 – Results (p. 15).

5 Results

5.1 Urinalysis of pregnant and non-pregnant alpacas (*Vicugna pacos*) and Bactrian camels (*Camelus bactrianus*)

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Journal of Camel Practice and Research

2018

Adopted from:

Kubátová A, Fedorova T. 2018. Urinalysis of pregnant and non-pregnant alpacas (*Vicugna pacos*) and Bactrian camels (*Camelus bactrianus*). Journal of Camel Practice and Research 25:303-306.

Authors' contributions:

The daft of the paper was written by KA with the usage of data from samples collected by KA and FT. KA and FT then finalized the paper in cooperation.

URINALYSIS OF PREGNANT AND NON-PREGNANT ALPACAS (*Vicugna pacos*) AND BACTRIAN CAMELS (*Camelus bactrianus*)

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ABSTRACT

The aim of this study was to test the possibility of using urinalysis in health control or alternatively for pregnancy diagnosis in non-invasively obtained samples from female alpacas and Bactrian camels kept in central Europe. Urine samples were collected from 12 female alpacas from three farms and from 14 female Bactrian camels from four zoos in the Czech and Slovak Republics. Samples were collected repeatedly at intervals of 4–9 weeks from 2010 to 2014. Spontaneous urination of animals was used to collect fresh urine samples into 0.5 L plastic cups held by hand or fastened to a telescopic rod. Immediately after sampling, the samples were tested using Duotest® double zone pH-indicator papers and DekaPhan® Leuco diagnostic test strips to obtain information about the specific gravity, the pH and the presence of leucocytes, nitrites, proteins, glucose, ketones, urobilinogen, bilirubin, blood and haemoglobin. In camels, urine colour was also observed. There were no problems with urine collections in the majority of animals thus non-invasive urine sampling was concluded as useful in camelids. However, none of the measured parameters showed a difference between pregnant and non-pregnant females (p > 0.05). The obtained results can serve as control values for urinalyses performed in camelids kept in small farms and zoos in the central Europe.

Key words: Alpacas, bactrian camel, urine, urinalysis

Alpacas and camels are important domestic animals in tropical and subtropical regions (Djemali and Alhadrami, 1998; Vilá Melo and Gutiérrez Vásquez, 2012), but they are also commonly kept as domestic or zoo-housed animals and pets (Gillespie and Flanders, 2010; Fowler, 2010). Urine testing can be useful not only for pregnancy diagnosis but also for health status monitoring (Czekala *et al,* 1990; Ganswindt *et al,* 2002; Rodríguez *et al,* 2017), because urinary pH can be used for the diagnosis of nutritional disorders (Nappert and Naylor, 2001) or post parturient diseases (Markusfeld, 1987). Urinary pH of healthy camelids ranges between 7.0 and 8.5; specific gravity from 1.018 to 1.050 (Cebra *et al,* 2013). According to a study by Banerjee *et al* (1981) urine of pregnant camels is darker, more alkaline (8.3 ±0.25 *vs.* 7.4 ± 0.37) and its specific gravity is higher than in non-pregnant female camels (1.086 ± 0.003 *vs.* 1.036 ± 0.01).

However, regardless of usefulness of urinalysis, laboratory methods are either unavailable or too expensive in some areas (Lanari *et al,* 2007), thus the data from urinalysis of camelids kept in small farms and zoological gardens of temperate regions are very limited or missing.

The aim of the present study was to test the possibility of using accessible diagnostic test strips intended for simple human urinalysis for basic non-invasive health control in female alpacas bred on small farms and zoo bred female Bactrian camels in two steps: 1) to compare the indicative results of urinalysis obtained *via* test strips designed for human use with the results obtained *via* more precise indicator papers; 2) to compare the indicative results of urinalysis with the health status of animals at the time of sampling. Additionally, the possibility to use basic urinalysis as a simple pregnancy diagnostic method in camelids was tested.

Materials and Methods

Urine samples were collected from both pregnant and non-pregnant 12 female alpacas (*Vicugna pacos*) and 14 female Bactrian camels (*Camelus bactrianus*) kept in 3 private farms and 4 zoos (Bratislava, Plzeň, Prague and Ústí nad Labem), respectively, in the Czech and Slovak Republics. In pregnant females, the urine was collected in different phases of pregnancy period. For each pregnancy, the date of parturition was recorded so the length of pregnancy could be calculated. In 1 alpaca female,

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abortion occurred during the research period so the date of parturition was estimated by a veterinarian according to the development of the foetus. Non-pregnant females were sampled repeatedly during different months of the year in the same time as pregnant animals.

In all alpacas, feeding was based on a combination of hay and pasture *ad libitum*, supplemented by recommended dosage of concentrated feed for alpacas. Camels were fed mainly by meadow hay *ad libitum*, supplemented by vegetables and pellets for herbivores, or cracked oats; fresh fodder was sometimes provided during the spring time or animals had access to grassy pasture. Fresh water was provided *ad libitum* in all breeding facilities.

Samples were repeatedly collected at intervals of 4–9 weeks during April 2013 – February 2014 for alpacas and September 2010 – November 2011 for camels. Urine samples were collected during spontaneous urination of animals throughout the whole day using 0.5 L plastic cups held in the hand or fastened to a telescopic rod (Fedorova *et al,* 2015).

Immediately after sampling, the pH was tested using Duotest® double zone pH-indicator papers (Macherey-Nagel GmbH and Co. KG, Germany). The specific gravity, pH and the presence of leucocytes, nitrites, proteins, glucose, ketones, urobilinogen, bilirubin, blood and haemoglobin was tested using DekaPhan® Leuco diagnostic test strips for urinalysis (Erba Lachema s.r.o., Czech Republic). Measurements obtained by DekaPhan® were considered approximate, because these test strips were designed for human use. In camels, urine colour, density and temperature were also evaluated, measured using a glass hydrometer and thermometer, respectively. The specific gravity was calculated from the urine density.

The data were analysed using the Statistica Cz 12 program (StatSoft, Inc., 2013).

Results and Discussion

There were no problems with urine collections in the majority of animals. Three alpacas (25%) and three camels (21%) were a little bit timid and urine collection was more complicated than in others. Total 60 samples from alpacas (31 from non-pregnant, 29 from pregnant females) and 62 from camels (21 from non-pregnant, 41 from pregnant females) were collected. The basic urine parameters measured in tested animals are shown in table 1. No significant difference was found (p > 0.05) between the pH as measured by DekaPhan® and Duotest® in either species, so we can conclude that it is adequate to use DekaPhan® for indicative measurement of pH values even though DekaPhan® is less precise than Duotest® due its less specific scale. All samples were within the interval considered normal for ruminant urinary pH (Sundra *et al,* 2004; Salles *et al,* 2012), with one single exception where a pH 5.5 sample was collected from a camel affected by diarrhoea.

Table 1. Basic urine parameters measured in female alpacas and Bactrian camels.

		Alpacas (n = 60)			Camels (n = 62)		
		Value	Min	Max	Value	Min	Max
Hydrometer	density [kg/m^3][a]	-	-	-	1,033.00 ± 2.60	1,010.00	1,080.00
	specific gravity[a]	-	-	-	1.03 ± 0.00	1.01	1.08
Duotest®	pH[a]	8.33 ± 0.06	7.00	8.80	8.42 ± 0.07	7.00	8.80
DekaPhan®	pH[a]	8.35 ± 0.10	6.00	9.00	8.50 ± 0.09	5.50	9.00
	specific gravity[a]	1.00 ± 0.00	1.000	1.015	1.00 ± 0.00	1.000	1.030
	leucocytes [leu/µl][b]	0	0	10-25	0	0	0
	nitrites [0, +, ++][b]	0	0	+	0	0	0
	protein [g/l][b]	0.30	0	5.00	0	0	5.00
	glucose [mmol/l][b]	0	0	0	0	0	55.00
	ketones [mmol/l][b]	0	0	1.50	0	0	1.50
	urobilinogen [µmol/l][b]	17.00	norm	17.00	norm	norm	17.00
	bilirubin [0, +, ++, +++][b]	+	0	+	0	0	+
	blood [ery/µl][b]	0	0	50.00	0	0	250.00
	haemoglobin [ery/µl][b]	0	0	0	0	0	250.00

[a] mean value±SE, [b] modus value, + light positive, Min minimum, Max maximum, n number of samples

Values for urinary pH (no matter which indicator papers were used), specific gravity and other urine parameters did not differ within alpaca farms (p > 0.05), except for proteins (p = 0.04). The urine of healthy alpacas should be negative for proteins (Fowler, 2010). However, proteins were detected in 35 urine samples of alpacas. Nevertheless, the results could be influenced by interference (Erba Group, 2015).

It is known that the pH of urine is influenced by the animal diet (Sundra *et al*, 2004), so the results are in accordance with the fact that the diets of alpacas were very similar within farms. On the other hand, in camels, both test strips showed differences in pH within zoos (p = 0.04 for both tests). However, multiple comparison tests showed no concrete differences between individual zoos in case of DekaPhan®. The results obtained *via* Duotest® suggested that the differences could be caused by different supplementary feeding in the form of grains or vegetable, not by green fodder. Also, the specific gravity as measured by DekaPhan® and the presence of ketones were influenced by the zoo in camels.

In both species, none of the measured parameters showed a significant difference between pregnant and non-pregnant females (p > 0.05), which supports the same conclusion derived from measurements of urinary pH of cattle by Veena and Narendranath (1993) and Dilrukshi and Perera (2009). But in the study of Skálová *et al* (2017), urinary pH was higher in pregnant heifers of cattle than in non-pregnant ones. Our results are incompatible with the hypothesis of Banerjee *et al* (1981) that pregnancy can be recognised by a change in the colour of camel urine.

In camels, the values of specific gravity obtained *via* hydrometer were higher than those obtained using the DekaPhan® strips (p < 0.01). However, DekaPhan® measurements can be influenced by pH values (Erba Group, 2015). The specific gravity obtained by hydrometer was always positively correlated with urinary pH, independent of whether Duotest® (R = 0.66, p < 0.01) or DekaPhan® (R = 0.38, p = 0.01) was used. Correlation between these parameters, even negative correlation, has been observed in humans (Shaafie *et al*, 2012). The specific gravity was also positively correlated with the protein content (R = 0.52, p < 0.01). The majority of the values obtained by hydrometer correspond with expected specific gravities for healthy camelids (Fowler, 2010). However, the results suggested that it is not possible to use urinary specific gravity for pregnancy diagnosis as was previously stated by Banerjee *et al* (1981).

In conclusion, it is possible to use accessible diagnostic test strips intended for simple human urinalysis for non-invasive indicative health control in camelids *via* selected parameters. Moreover, our findings do not support previously published results about usage of urinalysis for pregnancy diagnosis in camels.

Acknowledgements

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5.2 Non-invasive pregnancy diagnosis from urine by the Cuboni reaction and the barium chloride test in donkeys (*Equus asinus*) and alpacas (*Vicugna pacos*)

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Polish Journal of Veterinary Sciences on September

2016

Authors' contributions:

The paper was written by KA and FT with the usage of data collected by SI and HL. The paper was finally reviewed and commented by all authors.

Original article

Non-invasive pregnancy diagnosis from urine by the Cuboni reaction and the barium chloride test in donkeys (*Equus asinus*) and alpacas (*Vicugna pacos*)

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Abstract

The aim of the research was to evaluate two chemical tests for non-invasive pregnancy diagnosis from urine, the Cuboni reaction and the barium chloride test, in donkeys (*Equus asinus*) and alpacas (*Vicugna pacos*). The research was carried out from April 2013 to September 2014. Urine samples were collected on five private Czech farms from 18 jennies and 12 alpaca females. Urine was collected non-invasively into plastic cups fastened on a telescopic rod, at 6-9 week intervals. In total, 60 and 54 urine samples from alpacas and jennies, respectively, were collected. The Cuboni reaction was performed by the State Veterinary Institute Prague. The barium chloride test was done with 5 ml of urine mixed together with 5 ml of 1% barium chloride solution. Results of the Cuboni reaction were strongly influenced by the reproductive status of jennies; the test was 100% successful throughout the second half of pregnancy. However, no relationship was found between the real reproductive status of alpaca females and results of the Cuboni reaction. It was concluded that the barium chloride test is not suitable for pregnancy diagnosis either in donkeys, due to significant influence of season on the results, or in alpacas, because no relationship between results of the test and the reproductive status of alpaca females was found. In conclusion, the Cuboni reaction has potential to become a standard pregnancy diagnostic method in donkeys.

Key words: chemical test, jennies, non-invasive, spontaneous urination, urine collection

Introduction

Pregnancy diagnosis is essential for better reproductive management in captive animals (Thomas et al. 2010). Urine sampling by free catch is a non-invasive research method (Cote 2014) that enables collection of urine without restraining or capturing animals (Waits and Paetkau 2005), and breeders can collect urine by themselves. Pregnancy diagnosis from urine can be done by hormone analysis of the urine, but these methods mostly require collection of multiple samples and laboratory testing which are not always

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available (Hodges et al. 2010). Moreover, results of these analyses are available with some delay (Simersky et al. 2007).

Donkeys (*Equus asinus*) and alpacas (*Vicugna pacos*) are important husbandry animals for certain regions (FAO 2015). Moreover, both species are also bred as hobby animals in Europe, Australia, USA and other countries (Webster 2011) and although they are not closely related species, there are some similarities in their reproduction. The oestrous cycle in donkey jennies is influenced by season (Blanchard et al. 1999); however, according to Contri et al. (2014), not as strongly as in horse mares. In jennies, oestrous can be present during whole year in Europe. Alpaca females also show reproductive seasonality in the Andean region but they act like non-seasonal breeders in conditions of the Northern Hemisphere (Sumar 1999). Adult alpaca females do not show a typical oestrous cycle. Instead, they have continuous oestrous with short periods of non-receptivity (Fernández-Baca 1993), and ovulation is induced by mating (Bravo et al. 1991, Gauly and Bourke 1997). A relatively long gestation is typical for both species. The mean length of gestation is 365 days in donkeys (McDonnell 1998, Galisteo and Perez-Marin 2010) and about 345 days in alpacas (Volkery et al. 2012).

Various methods of pregnancy diagnosis are known in donkeys and alpacas but many of them are invasive, e.g. trans-rectal palpation (Rota et al. 2012, You et al. 2013) or measurement of hormonal levels in blood (Volkery et al. 2010, Crisci et al. 2014). Trans-abdominal ultrasonography (TAU) and male teasing are the most common non-invasive methods of pregnancy diagnosis in donkeys (Crisci et al. 2014) and alpacas (Volkery et al. 2010). However, an experienced sonographer (Rizk 2010) and relatively expensive equipment (Ndu et al. 2000a) are needed for TAU. Besides this, the method is time-consuming and requires clipping of a large portion of hair (Anderson et al. 2013). In the case of male teasing, the diagnosis can be done by breeders themselves but it is dependent on owning male individuals.

Because of this, there is a need for reliable, simple, affordable and non-invasive pregnancy diagnostic methods for alpacas and donkeys. For horse mares (*Equus caballus*) and donkeys, on-farm kits like the Wee-Foal-Checker exist, which are based on detection of a pregnancy-associated oestrogenic steroid metabolite in urine (Purohit 2010). Use of commercial urinary kits for donkeys is not always possible because of market inaccessibility in some countries and the relatively high price for one test. Nevertheless, other pregnancy tests from urine exist, such as chemical tests like the Cuboni reaction (Cuboni 1934) and the barium chloride test (Maslov and Smirnov 1965).

Both are probably based on the reaction of oestrogens with chemical substances (Stevenson 1945, Maslov and Smirnov 1965).

According to the authors' information, there is no available data on urinary oestrogen levels in jennies. However, blood levels are available. Concentrations of total oestrone remain <1ng/ml until the sixth week of pregnancy, then they increase to 600-2700 ng/ml during midpregnancy and again decrease to 1-20 ng/ml during the last two weeks of pregnancy (Hoffmann et al. 2014).

Concerning urinary oestrogen levels in alpacas, oestrone sulphate (E1S) concentration peaks twice during pregnancy. According to Bravo et al. (1996), the first peak is reached 21 days after mating, but the possibility of diagnosing pregnancy by the first peak of E1S was doubted by Volkery et al. (2012). The second peak is reached during the last month of gestation (Bravo et al. 1996, Volkery et al. 2012), when E1S concentrations reach 104.03 ± 24.09 ng E1S/mg creatinine. During the rest of the pregnancy, the average level is 8.82 ± 1.40 ng E1S/mg creatinine, which is very similar to the level in non-pregnant females (6.14 ± 0.53 ng E1S/mg creatinine) (Volkery et al. 2012).

The Cuboni reaction, which was developed for pregnancy diagnosis in horse mares (Cuboni 1934), is a test based on the colour reaction of free oestrogens in urine with sulphuric acid (H_2SO_4). In the urine of pregnant females, green opalescence should be detected after the Cuboni reaction. In the urine of non-pregnant females, a brown-red colouring should be visible (Stevenson 1945). According to Hayes (2002), it is possible to detect pregnancy in mares by the Cuboni reaction from 120 days after the conception. To get reliable results, it is better to wait until the 150th day of pregnancy, i.e. to the time of peak concentration 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). The Cuboni reaction has not been tested either in other *Equus* species or in South American camelids (*Lama* sp. or *Vicugna* sp.) but it has already been used in camels which are closely related to alpacas (Groves and Grubb 2011). In Bactrian camels (*Camelus bactrianus*) the Cuboni reaction was successful in the final third of pregnancy, when accuracy was 100% (Fedorova et al. 2015). In Arabian camels (*Camelus dromedarius*), sure positive reactions were obtained from slaughtered females with foetuses that had a crown vertebral rump length in the range 60-120 cm (El-Ghannam et al. 1974), which is reached during the second third of pregnancy (Bello et al. 2014).

The barium chloride test was developed by Maslov and Smirnov (1965), who discovered that addition of

a 1% solution of barium chloride ($BaCl_2$) to urine of non-pregnant cows (*Bos taurus*) causes creation of a white precipitate. In contrast, there is no reaction after addition of 1% $BaCl_2$ to the urine of pregnant cows. Even though the test is 50 years old, the factors preventing or inducing precipitation have not yet been discovered (Ndu et al. 2000a, Ndu et al. 2000b, Lalrintluanga and Dutta 2009). Oestrogens and progesterone are suspected as factors that can prevent precipitation (Maslov and Smirnov 1965, Lalrintluanga and Dutta 2009), but there are also researchers who do not agree (Ndu et al. 2000a,b). Lalrintluanga and Dutta (2009) reported the examination of this test in mares, but the results are not known. In Bactrian camels, the potency of the barium chloride test was not confirmed (Fedorova et al. 2015). However, according to Banerjee (1974), reliability was 85% between 50th and 90th day of pregnancy in Arabian camels (Purohit 2010).

The aim of this research was to evaluate the barium chloride test and the Cuboni reaction as pregnancy diagnostic tests for possible use in alpacas and donkeys.

Materials and Methods

Animals

Urine was collected from 18 jennies (*Equus asinus*) from two private Czech farms and from 12 alpaca females (*Vicugna pacos*) of huacaya type from three other private Czech farms. All alpaca females and 16 of the jennies were in their reproductive period; two jennies were still foals. The date of parturition was recorded for each pregnancy, and the sampling period in females was expressed as the number of days before parturition. Moreover, the date of the last successful mating as the day of probable conception was obtained from the breeders in the case of 11 pregnancies in donkeys and four pregnancies in alpacas and so the length of pregnancy was also calculated. An abortion occurred in one alpaca female during the study period and the date of parturition was estimated by a veterinarian according to the development of the foetus.

Urine sampling and processing

Urine samples were collected repeatedly from pregnant and non-pregnant animals at 6-9 week intervals from April 2013 to September 2014 and, in total, there were from one to five samplings from each jenny and five samplings from each alpaca female. The sampling period was from 34 to 363 and from 22

to 343 days before parturition in pregnant jennies and pregnant alpaca females, respectively. Non-pregnant animals were sampled repeatedly during different parts of the year. Urine was caught during spontaneous urination of the animals in half-litre plastic cups that were fastened onto a telescopic rod (Fedorova et al. 2015) or held in the hand in the case of tamed animals. Collected urine was poured into plastic test tubes (20 ml volume) for the purpose of transport and storage. Samples were refrigerated (Fedorova et al. 2015) at 5-7 ºC until testing. Before testing, urine was kept outside the refrigerator to reach room temperature and was then homogenised.

Cuboni reaction

Samples for the Cuboni reaction were transported to the State Veterinary Institute (SVI) Prague within one week after sampling and the reaction was done there by the method standardised for horse mares. The procedure of the Cuboni reaction in the SVI Prague was following: Urine was filtrated through filter paper and 5 ml of filtrated urine was mixed with 1 ml of concentrated hydrochloric acid (HCl) in a test tube. Then, the test tube was put into a boiling water bath for 10 min. After cooling, 6 ml of toluene was added and the solution was stirred properly. After 1 min, the two layers of the solution were divided. The lower layer with toluene was separated and filtrated into a clear test tube containing 1 ml of concentrated H_2SO_4. The mixture was stirred properly again and put into an 80 ºC water bath for approximately 15 min. After cooling, the colour of opalescence in the test tube placed on a dark background was evaluated.

Barium chloride test

Barium chloride tests were carried out in the laboratory of the Department of Animal Science and Food Processing, Czech University of Life Sciences Prague (CULS Prague) within one week after collection. In a test tube, 5 ml of urine was mixed with 5 ml of a 1% $BaCl_2$ solution (Krishna Rao and Veena 2009, Lalrintluanga and Dutta 2009). Then, the solution was agitated and left to stand for 5 min (Ndu et al. 2000a,b); after this, results were evaluated. Samples which showed any degree of cloudiness or turbidity after 5 min were regarded as having shown precipitation with the reagent and the test was concluded as negative (animal should be non-pregnant). When no reaction occurred in 5 min, the test was concluded as positive (animal should be pregnant) (Ndu et al. 2000a,b).

Table 1. Results of the Cuboni reaction in non-pregnant and pregnant jennies in the first and second halves of pregnancy. The results were significantly affected by the reproductive status (p<0.01).

Result of the Cuboni reaction	Reproductive status of females		
	Non-pregnant (n = 23)	1/2 pregnancy (n = 14)	2/2 pregnancy (n = 17)
Positive	13.04%	64.29%*	100.00%*
Negative	86.96%*	35.71%	0.00%

* An asterisk marks the correct results of the test (the result of the test correspond with the reproductive status of female).

Table 2. Results of the Cuboni reaction in non-pregnant and pregnant jennies in the first, second and final thirds of pregnancy. The results were significantly affected by the reproductive status (p<0.01).

Result of the Cuboni reaction	Reproductive status of females			
	Non-pregnant (n = 23)	1/3 pregnancy (n = 13)	2/3 pregnancy (n = 13)	3/3 pregnancy (n = 10)
Positive	13.04%	50.00%*	92.31%*	100.00%*
Negative	86.96%*	50.00%	7.69%	0.00%

* An asterisk marks the correct results of the test (the result of the test correspond with the reproductive status of female).

Table 3. Results of the Cuboni reaction in non-pregnant and pregnant alpaca females. The results were not significantly affected by the reproductive status (p>0.05).

Result of the Cuboni reaction	Reproductive status of females	
	Non-pregnant (n = 30)	Pregnant (n = 28)
Positive	73.33%	64.29%*
Negative	26.67%*	35.71%

* An asterisk marks the correct results of the test (the result of the test correspond with the reproductive status of female).

Statistical analysis

In cases where the last mating date was unknown, 365 and 345 days were considered as the mean lengths of pregnancy in donkeys (McDonnell 1998, Galisteo and Perez-Marin 2010) and alpacas (Volkery et al. 2012), respectively. Samples that were collected earlier than 365 and 345 days before parturition in jennies and alpacas, respectively, were considered as samples from a period of non-pregnancy. Postmature birth occurred in one alpaca female so the length of gestation of this female was not included in the general analysis.

The accuracy of the barium chloride test and Cuboni reaction were determined by comparison of the results with the real reproductive status of females which was either pregnant or non-pregnant. The results of the tests, which correspond with the reproductive status of females, were assessed as the correct results. Other results were evaluated as false-positive or false-negative. However, the reliability of individual tests in pregnant females was counted not only for the whole pregnancy period, but also for its halves and thirds. In alpacas, the analysis was also divided into the first month (first 31 days) of pregnancy, middle part (from 32 to 314 days) of pregnancy and the last month (last 31 days) of pregnancy. Additionally, the first 314 days and last month (31 days) of pregnancy were also tested separately using the Cuboni reaction, because of the changes in E1S levels during these phases described by Volkery et al. (2012).

In samples from non-pregnant females, the influence of the season on the results of both tests was analysed. 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) (Barnett and Dobson 2010). When the influence of seasons was not proved, data were assessed together. When results were influenced by season, data were evaluated separately for each season.

Data were statistically evaluated in the Statistica Cz 12 program (StatSoft, Inc., 2013). For all calculations, a significance level of $\alpha=0.05$ was established. All calculated numerical values were rounded off to two decimal places. To analyse the data, Pearson's chi-squared test was used.

Results

In total, we collected 114 urine samples from pregnant (n=31) and non-pregnant (n=23) jennies and from pregnant (n=29) and non-pregnant (n=31) alpaca females. The mean ($\pm$ SE) length of gestation was 361.36 $\pm$ 3.37 days in jennies and 338.00 $\pm$ 8.02 days in alpaca females.

Cuboni reaction

The results of the Cuboni reaction in non-pregnant jennies were not influenced (p>0.05) by the season. The results of the reaction showed significant differences (p<0.01) between non-pregnant and pregnant jennies when the pregnancy was considered as a whole period, divided into halves and even thirds (see Tables 1 and 2 for details). In pregnant jennies, 83.87% of samples were true positives and 16.13% false negatives. In non-pregnant jennies, 86.96% were true negatives and 13.04% false positives.

The results of the Cuboni reaction were also not influenced by the season (p>0.05) in non-pregnant alpaca females. No relationship was found between the real reproductive status of alpaca females and results of the Cuboni reaction, even if the accuracy was assessed for non-pregnancy versus pregnancy, halves of pregnancy or thirds of pregnancy (p>0.05). The results of the Cuboni reaction in both pregnant and non-pregnant alpaca females were mostly positive (see Table 3). The assessment of pregnancy stages when the first and last months of pregnancy were counted as extra stages was also unsuccessful (p>0.05). However, 3 out of 4 samples from alpacas in the last month of pregnancy showed a positive reaction.

Barium chloride test

A significant difference was found between the results of the barium chloride test in pregnant and non-pregnant jennies (p<0.05). Because the season had an influence on the results of the barium chloride test in non-pregnant jennies (p=0.03), the reliability of the barium chloride test was also counted separate-

ly for different seasons. In this case, results for pregnant jennies did not significantly differ from results for non-pregnant jennies during spring and autumn (p>0.05). Nevertheless, a difference was found in the summer season (p<0.01), when the urine of pregnant jennies (n=12) showed a predominantly negative reaction (91.67%); conversely, samples from non-pregnant females (n=8) reacted mainly positively (87.50%). When the pregnancy period of jennies was divided into halves and thirds and the results of the barium chloride test in these stages of pregnancy were compared to each other and to the results in non-pregnant jennies, no significant relationship was found between these groups (p>0.05).

The results of the barium chloride test were not influenced by the season (p>0.05) in non-pregnant alpaca females. Similarly to the Cuboni reaction, no significant relationship (p>0.05) was found between the reproductive status of alpaca females and results of the barium chloride test. The majority (92.86%) of urine samples showed a negative reaction in alpacas. Just 4 positive reactions (7.14%) occurred, 2 in pregnant and 2 in non-pregnant alpacas.

Discussion

The mean lengths of gestation in jennies and alpaca females in our study correspond substantially with other studies (McDonnell 1998, Galisteo and Perez-Marin 2010, Volkery et al. 2012).

The Cuboni reaction seems to be a suitable pregnancy diagnostic test for donkeys. Results in jennies are comparable with results in mares (Cuboni 1934, Hayes 2002, England 2008). These results were expected, because donkeys belong to the same genus, *Equus*, as horses (Groves and Grubb 2011). The Cuboni reaction could be also tested in other equid species, like zebras or Przewalski's horse, or even in other odd-toed ungulates as a non-invasive method of pregnancy detection. The collection of urine in odd-toed ungulates by the similar method as in presented study was already confirmed by Ramsay et al. (1994). The pregnancy diagnosis in equids is very important for management of captive animals. E.g. the introduction of a stallion, which was not sharing an enclosure with the mare in time close to conception, highly increases the risk of abortion or occurrence of infanticide after parturition in equids (Pluháček and Bartoš 2000, Gray 2009, Bartoš et al. 2011). The pregnancy diagnosis could help to prevent such events.

In comparison with Bactrian camels (Fedorova et al. 2015), the potential of using the test in donkeys is better. Even in camels, the reaction showed 100% reliability in pregnancy indication during the final third

of pregnancy (Fedorova et al. 2015); in jennies, the reliability also reached 100% throughout the whole second half of pregnancy. This corresponds to the fact that Cuboni reaction is based on reaction of oestrogens (Stevenson 1945) and the concentrations of these hormones increase from midpregnancy in jennies (Hoffmann et al. 2014).

Because of this, the Cuboni reaction has the potential to become a standard laboratory method of pregnancy diagnostic in donkeys, similar as in breedings of horses where the rectal palpation or other methods are difficult (Hayes 2002, England 2008). Opposite to that, the Cuboni reaction does not seem applicable in alpacas. Similar results with many false-positive and false-negative reactions were obtained in pigs (*Sus scrofa domestica*) (Heller 1940) and cattle (O'Moore 1947). The species most closely related to alpacas is the Bactrian camel (Janis et al. 1998), on which the Cuboni reaction was successfully tested (Fedorova et al. 2015). The failure of this method in alpacas should not be caused by a different way of storing and processing of samples or different evaluation of results because the urine samples of Bactrian camels collected by Fedorova et al. (2015) were also stored in the refrigerator before processing and also subjected to the Cuboni reaction at SVI Prague. We can also exclude the possible contamination of urine by soil, because it was caught directly into the cup, however alpacas sometimes defecated during urination and the faeces fell into the cup together with urine. The influence of the animals' management is improbable since animals were kept on three different farms.

False-negative results probably occurred in alpacas because the Cuboni reaction is based on the reaction of free oestrogens in urine (England 2008), and the urinary oestrogen level is highly increased just during the last month of pregnancy in alpacas (Bravo et al. 1996, Volkery et al. 2012). However, in alpacas, the test was not 100% successful even in the last month of pregnancy when the E1S levels reach a maximum (Bravo et al. 1996, Volkery et al. 2012). This could be caused by the very low number of samples from pregnant females in the last month of pregnancy (n=4) but pregnancy diagnosis during last month of pregnancy dwindles in importance. Besides this, the results in both non-pregnant and pregnant alpacas were mostly positive which suggests that the urinary oestrogen level was sufficient for obtaining positive results even in non-pregnant females. However, blood oestrogen levels of pregnant alpacas are not higher than in donkeys (Aba et al. 1998, Meira et al. 1998) and so no logical explanation of why the Cuboni reaction reaches mostly positive results in alpacas was found. It could be that some unknown

component of alpaca urine reacts positively during the Cuboni reaction.

In donkeys, the application of the barium chloride test seems controversial due to the influence of the season. The only significant differences were reached during the summer season but the results showed the opposite trend to other studies, e.g. Ndu et al. (2000a,b) or Lalrintluanga and Dutta (2009). However, similar opposite results have already been presented by Fedorova et al. (2015). Seasonal changes in oestrone levels in non-pregnant females could influence the results of the barium chloride test in jennies. However, according to Galisteo and Perez-Marin (2010), the main reproductive season of donkeys takes place in spring, when the majority of total foal-heat pregnancies were reported, not in summer when we reached only significant differences between pregnant and non-pregnant jennies by the barium chloride test. Besides this, the jennies included in the study gave birth throughout the year so we can cast doubt upon the influence of the season as in the study of Contri et al. (2014). Moreover, urinary oestrone conjugate levels of non-pregnant horse mares in heat never reach the levels in pregnant mares (Daels et al. 1991), and the general hormonal profiles of jennies are similar (Meira et al. 1998).

It was concluded that the barium chloride test is not a suitable pregnancy diagnostic test in alpacas, similar to recent findings for cattle (Krishna Rao and Veena 2009) and Bactrian camels (Fedorova et al. 2015). The urine of alpaca females almost always coagulated after addition of 1% $BaCl_2$ solution. In contrast, there have been successful experiments in pigs (Ndu et al. 2000a,b, Lalrintluanga and Dutta 2009) and Arabian camels (Banerjee 1974, Purohit 2010).

We tend to agree with the conclusion of Ndu et al. (2000a,b) that differences in results between pregnant and non-pregnant females are probably not caused by progesterone (P4) and pregnanediol-3-glucuronide (PdG). Average differences in P4 urinary levels in non-pregnant and pregnant alpaca females are high (Volkery et al. 2012) and thus no difference between results of the barium chloride test in pregnant and non-pregnant female alpacas indicates that neither P4 nor PdG are factors preventing or inducing precipitation.

Nevertheless, we must admit that oestrogens do not seem likely to be preventing or inducing factors of precipitation even in jennies. If we know that total blood oestrone reaches maximal levels during mid-pregnancy and maintains this level until two weeks before parturition (Hoffmann et al. 2014), we would expect that the test would work during the second half or at least the final third of pregnancy in jennies. Nevertheless, testing of the reliability for halves and

thirds of pregnancy was not successful. In conclusion, oestrogens are probably not preventing or inducing factors of precipitation in the barium chloride test. This statement can also be supported by the fact that we reached different results in the Cuboni reaction which is demonstrably based on reaction of oestrogens (Stevenson 1945).

It is not certain what causes the precipitation of alpaca urine. An increased number of crystals was observed in alpaca urine samples after refrigeration. This was also described in the urine of cats and dogs by Albasan et al. (2003), who found that an increased number and size of crystals was caused by refrigeration, and the number of crystals was higher the longer the refrigeration time. These crystals could be the factor which caused the precipitation of almost all urine samples in alpacas, because urine was not filtrated before barium chloride test processing. Even though the filtration of urine before barium chloride test processing was not described by any author that was cited in this paper, they usually tested freshly collected urine (Ndu et al. 2000a,b, Krishna Rao and Veena 2009, Lalrintluanga and Dutta 2009). Due to the lack of information about factors that influence test results, there is also a lack of information about how urine should be properly preserved for this test. However, the samples of donkey urine were preserved in the same way and the precipitation of urine did not occur so often even though the samples were not filtrated either.

The unreliable results of barium chloride test can be affected also by different content of analytes in urine of studied species. Chemical composition of urine can be different across species. Although camelids and bovids are both ruminants, the anatomy and physiology of digestive tract is different in some aspects (Fowler 2010). Moreover diet and feeding management also affects the composition of urine (Tamminga 1992, Hu and Murphy 2004). According to Maslov and Smirnov (1965), high accuracy of barium chloride test was obtained just in cows kept in cow houses. Animals on pasture, as in our study, could have higher urine oestrogen level due to presence of phytoestrogens in the pasture (Maslov and Smirnov 1965).

In conclusion, the Cuboni reaction seems applicable for pregnancy diagnosis in jennies. The Cuboni reaction has many advantages: (i) collection of urine can be done by breeders themselves, (ii) the reaction can be carried out by accredited laboratories and (iii) the price is acceptable. Because of this, the Cuboni reaction has the potential to become a standard pregnancy diagnostic method in donkeys. In contrast, neither the Cuboni reaction nor the barium chloride test were confirmed as suitable pregnancy diagnostic methods for alpacas, and a search for other simple non-invasive methods for pregnancy diagnosis in alpacas is needed.

Acknowledgments

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5.3 Seed germination test as an alternative urine-based non-invasive pregnancy test in alpacas (*Vicugna pacos*)

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Journal of Camel Practice and Research

2016

Adopted from:

Kubátová A, Fedorova T. 2016. Seed germination test as an alternative urine-based non-invasive pregnancy test in alpacas (*Vicugna pacos*). Journal of Camel Practice and Research 23:261-264.

Authors' contributions:

The daft of the paper was written by KA with the usage of data from samples collected by KA. KA and FT then finalized the paper in cooperation.

SEED GERMINATION TEST AS AN ALTERNATIVE URINE-BASED NON-INVASIVE PREGNANCY TEST IN ALPACAS (*Vicugna pacos*)

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ABSTRACT

The goal of this study was to assess the seed germination test as a pregnancy diagnostic method in alpacas (*Vicugna pacos*). Sampling was carried out in 6-8 week intervals from April 2013 to February 2014 on three private farms in the Czech Republic (Central Europe). The urine was collected non-invasively by catching it in plastic cups during spontaneous urination. In total, five urine samples were obtained from each of the 12 tested alpacas. Two urine concentrations were tested, and the urine was diluted by distilled water in ratios of 1:4 and 1:14. Fifteen millilitres of the urine-water solution was applied onto 50 mung bean (*Vigna radiata*) seeds in Petri dishes. Germination rates were counted two days after establishment of the experiments. Lengths of the shoots were measured on the fifth day. It was determined that alpaca urine significantly inhibited germination and growth of seeds in general. The inhibitory effect was higher with higher concentrations of urine. However, seeds germinated and grew better in the urine of pregnant females than in urine of non-pregnant females. While further research is needed, the seed germination test with mung beans seems to be a viable method for pregnancy diagnosis in alpacas.

Keywords: Mung bean, pregnancy diagnosis, Punyakoti test, shoot length, *Vigna radiata*

The predecessor of the seed germination test, which is also called the Punyakoti test (Veena Ganesaiah, 2006) was described in the ancient Egyptian papyrus approximately 4,500 years ago. The test was used in women and it 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).

In an attempt to find a new simple test for pregnancy diagnosis in cattle, this test was later examined in cows (*Bos taurus*). It was discovered that the germination and growth of wheat seeds were inhibited by the urine of pregnant cows significantly more so than by the urine of non-pregnant cows (Veena and Narendranath, 1993), which is a completely opposite reaction to that found in humans (Veena Ganesaiah, 2006).

The majority of related studies have determined that the rates of germination and growth of seeds treated with urine were significantly lower than that of seeds treated with distilled water (Ghalioungui *et al,* 1963; Veena and Narendranath, 1993; Dilrukshi and Perera, 2009; Narayana Swamy *et al,* 2010). Nevertheless, Rine *et al* (2014) did not find a significant difference between treating with distilled water and the urine of non-pregnant cows and moreover, between distilled water, urine of non-pregnant cows and urine of pregnant cows up to 21 days of pregnancy.

The test was later extended to buffaloes (*Bubalus bubalis*) (Veena *et al,* 1997; Dilrukshi and Perera, 2009), domestic sheep (*Ovis aries*) and goats (*Capra hircus*) (Veena Ganesaiah, 2006; Rao Krishna and Veena, 2009) and mithuns (*Bos frontalis*) (Perumal, 2014). In all of these animals, the inhibition effect on seeds was higher with the urine of pregnant females than with the urine of non-pregnant females.

Besides wheat seeds (Veena and Narendranath, 1993; 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 processing.

The cause of the difference in germination between seeds in urine of pregnant and non-pregnant animals has not yet been determined. The following chemical substances and factors have been considered as culprits: pH (Veena and Narendranath,

1993), animal hormones (Nirmala *et al,* 2008) or their metabolites (Rao Krishna and Veena, 2009) and plant hormones such as auxins (Veena and Narendranath, 1993) or abscisic acid (ABA), which can cause dormancy of seeds (Veena Ganesaiah, 2006).

The seed germination test has many positive attributes, such as its non-invasiveness, simplicity, safety, acceptable cost, high reliability and practicability as it can be performed directly on farms and by farmers themselves (Rao Krishna and Veena, 2009; Narayana Swamy *et al,* 2010; Rine *et al,* 2014). The negative attributes are the time demands and relative laboriousness (Krishna Rao and Veena, 2009).

The goal of this research was to assess the seed germination test as a non-invasive pregnancy diagnostic method in alpacas (*Vicugna pacos*). Our hypothesis was that the urine of pregnant female alpacas will inhibit the seed germination and shoot length more so than the urine of non-pregnant female alpacas.

Materials and Methods

Animals

Samples of urine were collected from 12 adult huacaya alpaca females (*Vicugna pacos*) from three private farms in the Czech Republic (Central Europe). For each pregnancy, the date of parturition was recorded, and in the case of four pregnancies, the date of the last successful mating as the day of probable conception was also known so the length of pregnancy could be calculated. In one alpaca female, an abortion occurred during the research period so the date of parturition was estimated by a veterinarian according to the development of the foetus.

Urine sampling

The research was carried out from April 2013 to February 2014 when urine was collected repeatedly from pregnant and non-pregnant females at 6-8 week intervals. In total, there were five samplings from each alpaca female adding up to 60 samples in total. In pregnant females, the sampling period was from 16 to 343 days before parturition. Non-pregnant females were sampled repeatedly during different parts of the year. Samples were collected during spontaneous urination of the females into plastic cups (0.5 L volume) fastened onto a telescopic rod (Fedorova *et al,* 2015). After collection, urine was poured into plastic test tubes (20 ml volume) for the purpose of transport from the farm. After homogenisation, the seed germination tests were immediately started.

Seed germination test

In this study, mung bean seeds (*Vigna radiata*) were used (Dilrukshi and Perera, 2009; Rao Krishna and Veena, 2009). The seeds were of the product line Albert Bio (Ahold Czech Republic, a.s.), which means they were of organic quality intended for human consumption, and they were purchased in the food store.

During the first step of processing, each urine sample was divided into two parts. One part was diluted with distilled water in the ratio 1:4 (1 part urine, 4 parts water) (Veena and Narendranath, 1993; Narayana Swamy *et al,* 2010) and the second part in the ratio 1:14 (1 part urine, 14 parts water) (Dilrukshi and Perera, 2009). Fifteen millilitres of each of the urine-water solutions were applied onto two sterile Petri dishes containing 50 seeds. This means that for each urine sample there were four Petri dishes containing 50 seeds, two of them filled by the 1:4 concentration samples and two by the 1:14 concentration samples.

A control test with just 50 seeds and 15 ml of distilled water was performed separately (Veena and Narendranath, 1993; Narayana Swamy *et al,* 2010).

The number of germinated seeds was counted 48 hours after the establishment of the experiments. In germinated seeds, the length of the shoots was measured in millimetres by a ruler on the fifth day (Veena and Narendranath, 1993; Rao Krishna and Veena, 2009).

All the tests were performed at room temperature (18-22°C) in a north-facing laboratory with windows, so natural daylight was present without direct sunshine. No artificial light was used.

Statistical analyses

When the last mating date was unknown, 345 days was considered as the mean length of pregnancy in alpacas (Volkery *et al,* 2012). Samples that were collected earlier than 345 days before parturition were considered as samples from a period of non-pregnancy.

The accuracy of the seed germination test was determined by comparing the results with the true reproductive status of the females (either pregnant or non-pregnant).

Data were statistically evaluated in the StatisticaCz 12 program (StatSoft, Inc., 2013). A significance level of α = 0.05 was established for all calculations. All calculated numerical values were rounded off to two decimal places. For analyses, non-

parametric Kruskal-Wallis tests followed by multiple comparisons tests were used, because the data were not normally distributed (Kolmogorov-Smirnov test, $p < 0.01$).

Results

In total 12,750 seeds were used for experimental purposes (750 seeds in control samples, 6,200 seeds in non-pregnant females and 5,800 seeds in pregnant females).

Two days after the establishment of the experiments, the seeds showed significant differences in germination rates according to the solution in which they were immersed ($p < 0.01$). Similar results were observed after the fifth day for shoot lengths ($p < 0.01$).

Experiments with 1:4 concentration of urine

For experiments with the 1:4 urine dilution, seeds from control tests that were treated only with water had higher germination rates than the seeds treated with urine, regardless of pregnant ($p < 0.01$) or non-pregnant females ($p < 0.01$). The seeds treated with urine of non-pregnant females had lower germination rates than the ones treated with urine of pregnant females ($p = 0.03$).

The same results were gained in shoot length measurements. The shoots of the seeds from control tests were longer than the ones from seeds treated with urine, of both pregnant ($p < 0.01$) and non-pregnant females ($p < 0.01$). The seeds treated by urine of non-pregnant females had the shortest shoots ($p < 0.01$). Detailed results of germination rates and shoot lengths of the seeds treated by the 1:4 urine

solution compared to control samples are shown in Table 1.

Experiments with 1:14 concentration of urine

The experiments with the 1:14 concentration of urine also showed significant differences among groups in germination rates ($p < 0.01$) as well as in shoot lengths ($p < 0.01$). However, regarding germination, the difference was in significantly higher germination rates of seeds in control tests compared to seeds treated by urine of non-pregnant females ($p < 0.01$). In contrast, germination rates of seeds treated by urine of pregnant females did not significantly differ from the other two groups: control tests ($p > 0.05$) and non-pregnant females ($p = 0.051$).

Nevertheless, the results of shoot lengths had the same trend and p-values as in the seeds treated with the 1:4 concentration of urine (Table 2).

Discussion

In general, the alpaca urine from both pregnant and non-pregnant females inhibited germination and growth of the mung beans, compared to seeds treated by distilled water. The same results were obtained with wheat seeds in cattle (Veena and Narendranath, 1993; Krishna Rao and Veena, 2009; Narayana Swamy *et al,* 2010) and humans (Ghalioungui *et al,* 1963) and with mung beans in cattle (Dilrukshi and Perera, 2009; Rao Krishna and Veena, 2009).

The inhibition effect of urine on germination and growth of mung beans was higher with the 1:4 concentration of urine than with the 1:14 concentration of urine. The weaker inhibition of the 1:14 solution was caused by a higher ratio of water, and these results

Table 1. Germination rates and shoot lengths of mung beans treated by water (control) and urine of pregnant and non-pregnant alpaca females in a 1:4 dilution. Means followed by the same letter in the same column were not significantly different (multiple comparisons test; $p > 0.05$).

Treatment	Germination rate (%)		Shoot length (mm)	
	Mean	SE	Mean	SE
Control (distilled water)	99.20[a]	0.26	42.39[a]	0.84
Urine of pregnant alpaca females	89.14[b]	3.20	8.22[b]	0.17
Urine of non-pregnant alpaca females	61.03[c]	6.72	4.83[c]	0.10

Table 2. Germination rates and shoot lengths of mung beans treated by water (control) and urine of pregnant and non-pregnant alpaca females in a 1:14 dilution. Means followed by the same letter in the same column were not significantly different (multiple comparisons test; $p > 0.05$).

Treatment	Germination rate (%)		Shoot length (mm)	
	Mean	SE	Mean	SE
Control (distilled water)	99.20[a]	0.26	42.39[a]	0.84
Urine of pregnant alpaca females	97.21[a,b]	0.67	25.64[b]	0.40
Urine of non-pregnant alpaca females	94.74[b]	1.01	14.65[c]	0.26

correspond with the findings of Dilrukshi and Perera (2009) who used mung beans in cattle.

Except for the results of seed germination in the 1:14 urine dilution where the results were not statistically significant, similar to results from Rine *et al* (2014), seeds always germinated and grew significantly better in the urine of pregnant females than in the urine of non-pregnant females. This exception might have been caused by excessive dilution of the urine, causing the "pregnancy factor" that affects seed growth to be lost.

Studies of human urine obtained the same results, where the urine of non-pregnant females inhibited the germination and growth of seeds more than that of pregnant females (Hoffmann, 1934; Ghalioungui *et al*, 1963). Even though alpacas are even-toed ungulates, similar to the other domestic animals in which this pregnancy test was assessed, the obtained results in alpacas were opposite to those from all other tested animals including cattle (Veena and Narendranath, 1993; Dilrukshi and Perera, 2009; Narayana Swamy *et al*, 2010), mithuns (Perumal, 2014), buffaloes, sheep and goats (Rao Krishna and Veena, 2009). Because of this, our hypothesis was rejected.

Further research should verify these findings and extend the experimental design. Further studies should also include observations of the changes of urine and seed colour, as was done by other authors (Krishna Rao and Veena, 2009; Rao Krishna and Veena, 2009). However, it seems that the seed germination test has potential to be a pregnancy diagnostic test in alpacas.

Acknowledgements

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5.4 Urinary reproductive hormones influence seed germination within diluted urine of heifers

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Authors' contributions:

The daft of the paper was written by LI with the usage of data from samples collected by LI and analysed by ŠM. KA and FT processed additional analyses and finalised the paper. KA and FT have also rewritten the paper for the submission to the other journal.

Urinary reproductive hormones influence seed germination within diluted urine of heifers

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ABSTRACT

Experimental non-invasive pregnancy diagnostic techniques, such as seed germination testing in diluted urine, may serve as an attractive alternative to standardized pregnancy diagnostic methods, but scientific validation of these methods and revelation of their principles is insufficient. This study aimed to use Czech Fleckvieh heifers to investigate the influence of urinary reproductive hormones (oestrone sulphate, 17β-oestradiol, and pregnanediol-3-glucuronide) on the germination success of seeds placed within their diluted urine, and further to verify the reliability of seed germination test for pregnancy diagnosis in this species. Mung bean and wheat seeds were germinated in two urine-water dilutions (1:4 and 1:14) for three days, using urine samples obtained from either pregnant or non-pregnant heifers. Germinated seeds were counted daily, and the shoot lengths were measured three days after placement in the urine. Levels of urinary reproductive hormones were determined using competitive heterogeneous enzyme immunoassays (EIAs). Despite the dilution rate used, final results indicated that the urine from pregnant heifers inhibited germination and growth in mung beans only. On the contrary, wheat germination rate was higher within the urine from pregnant females on days 1 and 2, when using a 1:14 dilution rate. For both seed species, correlations between urinary hormone profiles and seed germination parameters were found, depending on the day of the experiment and the rate of dilution used. The shoot lengths of mung bean seeds were significantly ($P < 0.05$) and negatively correlated with the profiles of all hormones analysed from the urine samples. Thus, urinary reproductive hormones influence seed germination and growth rate of wheat and mung beans, indicating the potential for this technique to be used for pregnancy determination in heifers.

Keywords: Germinated seeds, non-invasive pregnancy diagnosis, *Triticum aestivum, Vigna radiata*

INTRODUCTION

Free catch of urine is a suitable way to non-invasively obtain urine of different species without the animal being restrained or sedated (Kubátová *et al.*, 2016). Urine samples can be processed by experimental diagnostic methods, such as the seed germination test (Narayana Swamy *et al.*, 2010) or reproductive hormone assays (Kirkpatrick *et al.*, 1992). The seed germination test compares shoot length and the number of germinated seeds in diluted urine of pregnant and non-pregnant females (Rine *et al.*, 2014). According to previous studies, the urine of pregnant cows significantly inhibits the germination and growth of seeds (Veena and Narendranath, 1993; Dilrukshi and Perera, 2009; Skálová *et al.*, 2017). However, factors influencing the differences in the germination of seeds in urine of pregnant and non-pregnant animals are still unknown. To the best of our knowledge, no studies have reported the relation between urinary reproductive hormones in cattle and the germination of seeds kept in diluted urine. To date, only one study has examined the effects of different concentrations of 17ß-oestradiol (E2) and progesterone (P4) on mung bean and wheat seeds and concluded that they do not influence seed germination rate (Nirmala *et al.*, 2008).

The determination of reproductive hormones in the urine by enzyme-immunoassay (EIA) has been described in several ungulate species (Kirkpatrick *et al.*, 1991; Volkery *et al.*, 2012). Pregnanediol-3-glucuronide (PdG) is a urinary metabolite of P4 (Loskutoff *et al.*, 1983) and was found at higher concentrations in pregnant cows than in non-pregnant ones with a peak on day 16 after artificial insemination (Yang *et al.*, 2004). While P4 remains at constant levels during cattle gestation and decreases approximately 10 days before parturition (Catchpole, 1969), the concentrations of plasma oestrone sulphate (E1S) and E2 increase progressively as pregnancy advances and reach peak levels before calving

(Desaulniers *et al.*, 1989; Shah *et al.*, 2006). The concentration of E1S increases earlier in the urine than in the blood of pregnant cows (Yang *et al.*, 2003).

This study investigated the influence of urinary reproductive hormones on the germination of seeds kept in diluted urine of cattle, which can serve as a model species for related wildlife such as buffaloes or antelopes. Moreover, it aimed to identify differences in seed germination rate in the urine of pregnant and non-pregnant heifers for possible pregnancy diagnosis. The results may help in implementing the seed germination test for better pregnancy diagnosis in small farms or breeding facilities where sophisticated laboratory equipment is not available or its use is limited due to limited possibilities of handling untamed animals.

MATERIALS AND METHODS

The research was carried out in accordance with the current legislation of the Czech Republic and the European Union and was approved by the management of the farm involved. According to Act no. 246/1992 Coll. of the Czech Republic and Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes, the study met all of the requirements of non-invasive research so no other ethical approval was needed. Animals were not forced to cooperate and did not suffer stress. They were accustomed to the sampling devices and sampling process step by step from the start of the research period.

Animals

Czech Fleckvieh heifers (n = 12 pregnant, n = 10 non-pregnant) from a dairy farm in Vendolí, the Czech Republic (49°44′10″N 16°24′45″E, 480 MASL), were randomly selected for the study. The heifers were fed with a total mixed ration composed of clover haylage, corn silage, straw, grain, and mineral supplements for heifers. All heifers were

free of reproductive and digestive disorders. Females were considered as pregnant when their pregnancy was confirmed by a veterinarian using transrectal ultrasonography (at the earliest 28 days after artificial insemination). All pregnancies were verified by a successful parturition as well. Non-pregnant heifers served as control animals, and their oestrous cycles were not monitored.

Urine sample collection

The research was carried out from May to December 2014. The urine of pregnant heifers was non-invasively collected at 2-week intervals from the 4th to 8th week of pregnancy. The urine of non-pregnant heifers was sampled at irregular intervals. Urine collection started after morning feeding, around 06:00 AM. Midstream urine was collected into half-litre plastic cups fastened on a telescopic rod during spontaneous urination (Haberová *et al.*, 2011). For the seed germination tests, urine was poured into closable plastic vials (20 or 60 ml), refrigerated at 5–7 °C, and transported to the laboratory within 12 hours (Haberová *et al.*, 2011). For the hormonal assays, urine was poured into three closable plastic Eppendorf tubes (3 or 5 ml), frozen at -20 °C (Volkery *et al.*, 2012) and transported within 12 hours to the laboratory in a car refrigerator surrounded by frozen gel to prevent the samples from defrosting. The samples were stored at -20 °C until hormone analyses (Volkery *et al.*, 2012).

Sample preparation for seed germination test

The germination tests were performed by diluting urine samples with distilled water at the ratio of 1:4 (1 part urine and 4 parts water) and 1:14 (1 part urine and 14 parts water) (Dilrukshi and Perera, 2009; Narayana Swamy *et al.*, 2010). Winter wheat (*Triticum aestivum*) and mung bean (*Vigna radiata*) seeds intended for human consumption and bought in a grocery store were used for this research. Seeds were prepared in sterile Petri dishes and the urine-water solution (20 ml) was applied onto the seeds. Four seed

germination tests, with 50 seeds in each sterile Petri dish, were performed for every urine sample: 1) mung beans treated with dilution 1:4; 2) mung beans treated with dilution 1:14; 3) wheat seeds treated with dilution 1:4; 4) wheat seeds treated with dilution 1:14. The prepared tests containing seeds together with the urine-water solution were kept under laboratory conditions for 3 consecutive days at a constant room temperature of around 25 °C under natural day light. The number of germinated seeds was counted daily, always at the same time of day, and the length of shoots was measured at the end of the test (day 3) using a ruler.

Control experiments were carried out with 20 ml of distilled water and 50 mung bean or 50 wheat seeds in each Petri dish (Veena and Narendranath, 1993; Narayana Swamy *et al.*, 2010) under the same conditions.

Urinary hormone assays

The urinary reproductive hormones (E1S, E2 and PdG) were determined by competitive heterogeneous enzyme immunoassay (EIA). The Estrone Enzyme Immunoassay Kit, 17-β-oestradiol Enzyme Immunoassay Kit and Pregnanediol-3-Glucuronide Enzyme Immunoassay (Arbor Assays, USA) were used. Sample preparation, reagent preparation and the assay protocol were performed according to the manufacturer's recommendations (DetectX®, Arbor Assays, USA). To determine the optimal concentration of diluted urine to use for the assay, we compared three dilution factors of our samples to a standard curve generated using positive control hormones supplied with the assay. Urine diluted with assay buffer (component of assay kit) was tested before the final urine hormone assay. Three dilution factors were prepared for each tested hormone to achieve the ideal dilutions, which were equivalent to the standards of each particular hormone. The dilution that was equivalent to the standards of a particular hormone (determined by Arbor Assays, USA) was used in the subsequent urine hormone examination. Standards were prepared

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by serial dilution of standard solution (i.e. solution with known concentration of given hormone). The final dilutions used for urinary E1S, E2 and PdG determination are displayed in Table 1.

Table 1: Dilution of urine samples with Assay Buffer

Hormone	Pregnancy status of heifers	Urine sample:Assay Buffer
Oestrone sulphate	Pregnant	1:12
	Non-pregnant	1:4
17β-oestradiol	Pregnant	1:1
	Non-pregnant	1:1
Pregnanediol-3-glucuronide	Pregnant	1:9
	Non-pregnant	1:4

The hormone concentrations were evaluated by measuring absorbance with a spectrophotometer (VersaMax ELISA reader, Molecular Devices, USA) at wavelength $\lambda = 450$ nm. A four-parameter logistic calibration curve was generated using standard samples. The concentrations of the hormones in the urine samples were determined from the standard curve using SoftMax Pro 5 software (Molecular Devices, USA).

Data analysis

Data were analysed using Statistica Cz 12 software (StatSoft, Inc., 2013). As the data for the number of germinated seeds did not show a normal distribution, the influence of pregnancy on the number of germinated seeds was statistically evaluated using the nonparametric Mann-Whitney U test. The influence of pregnancy on the length of shoots was evaluated by ANOVA. The differences between concentrations of reproductive

hormones in pregnant and non-pregnant heifers were evaluated using the Mann-Whitney U test. The relationship between reproductive hormones and the number of germinated seeds as well as the lengths of shoots was tested using the Spearman's rank correlation. Significance was accepted at 0.05.

RESULTS

Totally 36 and 50 urine samples were obtained non-invasively from pregnant and non-pregnant heifers, respectively. In total, 8,600 mung beans and 8,600 wheat seeds were processed in the seed germination tests, among which 4,844 mung beans and 1,929 wheat seeds germinated.

Number of germinated seeds

The presence of heifers' urine, regardless of pregnancy status, inhibited the number of mung bean and wheat seeds that germinated in both dilutions compared to the control conditions containing distilled water for all three days of the test ($P < 0.01$ for all cases). Pregnancy status did not significantly ($P > 0.05$) influence the number of mung bean seeds that germinated in both dilutions on days 1 and 2 of the experiment. On day 3, a significantly lower number of germinated mung bean seeds was counted in urine obtained from pregnant heifers than in non-pregnant heifers for both dilutions ($P = 0.020$ and $P = 0.015$ for dilution 1:4 and 1:14, respectively).

The results for wheat seeds showed the opposite trend. No significant differences were observed in the number of wheat seeds that germinated in urine of pregnant and non-pregnant heifers when exposed to 1:4 dilution ($P > 0.05$ for all three days) whereas significant differences were observed when wheat seeds were exposed to a 1:14 dilution on day 1 ($P = 0.001$) and day 2 ($P = 0.031$) of the experiment. For detailed results see Table 2.

Table 2: Mean number of germinated mung beans and wheat seeds kept in urine diluted 1:4 and 1:14 during all three days of the test. Significance level indicates statistical differences between pregnant and non-pregnant heifers.

Test	Day	Pregnant heifers		Non-pregnant heifers		Significance level
		Mean	SE	Mean	SE	
Mung beans dilution 1:4	1	12.944	1.632	13.040	1.173	P > 0.05
	2	15.389	1.687	19.520	1.674	P > 0.05
	3	16.528	1.815	22.540	1.788	P < 0.05
Mung beans dilution 1:14	1	25.028	1.823	21.980	1.631	P > 0.05
	2	30.306	1.582	32.940	1.710	P > 0.05
	3	32.639	1.667	37.320	1.727	P < 0.05
Wheat seeds dilution 1:4	1	7.250	0.798	5.480	0.533	P > 0.05
	2	8.278	0.862	6.700	0.582	P > 0.05
	3	8.556	0.889	7.180	0.628	P > 0.05
Wheat seeds dilution 1:14	1	14.306	1.087	10.160	0.589	P < 0.01
	2	15.333	1.093	12.520	0.764	P < 0.05
	3	15.778	1.084	13.880	0.906	P > 0.05

Length of shoots

The length of shoots was also inhibited by the presence of heifers' urine, regardless of pregnancy status. Significantly shorter shoots were measured in mung bean seeds that germinated in urine-water solutions (P < 0.001 for both dilutions) compared to distilled

water only. Similar findings were observed in wheat seeds (P < 0.001 and P < 0.01 for dilution 1:4 and 1:14, respectively).

Significantly shorter shoots were measured in mung bean seeds that germinated in the urine of pregnant heifers (P < 0.0001 for both dilutions). No significant effect (P > 0.05) of pregnancy status on shoot length was observed in wheat at either dilution of urine.

Hormonal profiles and their relationships to seed germination rate and shoot length

The mean concentrations of urinary E1S, E2 and PdG in pregnant and non-pregnant heifers are displayed in Table 3. All results differed significantly between pregnant and non-pregnant heifers (P<0.001).

Table 3: Mean ± SE concentrations of urinary oestrone sulphate, 17β-oestradiol and pregnanediol-3-glucuronide. All results differed significantly between pregnant and non-pregnant heifers (P<0.001).

Hormone	Pregnant heifers	Non-pregnant heifers
Oestrone sulphate (pg/ml)	2477.186 ± 107.826	808.929 ± 59.871
17β-oestradiol (ng/ml)	13.019 ± 0.830	2.505 ± 0.180
Pregnanediol-3-glucuronide (ng/ml)	225.520 ± 23.698	26.650 ± 1.683

The detailed correlations between measured concentrations of reproductive hormones and numbers of germinated seeds are displayed in Table 4.

Table 4: The correlations between concentrations of reproductive hormones and numbers of germinated seeds.

Test	Day	Oestrone sulphate	17β-oestradiol	Pregnanediol-3-glucuronide
		r	*r*	*R*
Mung beans dilution 1:4	1	0.005	-0.107	0.061
	2	-0.207	-0.241*	-0.146
	3	-0.269*	-0.284*	-0.210
Mung beans dilution 1:14	1	0.086	-0.080	0.053
	2	-0.140	-0.321*	-0.227*
	3	-0.235*	-0.361*	-0.352*
Wheat seeds dilution 1:4	1	0.096	0.125	0.339*
	2	0.038	0.088	0.286*
	3	0.05	0.093	0.296*
Wheat seeds dilution 1:14	1	0.242*	0.416*	0.381*
	2	0.132	0.372*	0.251*
	3	0.043	0.294*	0.175

Legend: * Significant correlation at P<0.05 level.

Weak but significant (P < 0.05) negative correlations were found between the length of mung bean shoots and concentrations of all measured reproductive hormones both for dilution 1:4 (*r* = - 0.139, *r* = - 0.111, and *r* = - 0.125 for E1S, E2 and PdG, respectively) and for dilution 1:14 (*r* = - 0.190, *r* = - 0.269 and *r* = - 0.274 for E1S, E2 and PdG, respectively).

In the case of wheat seeds treated with dilution 1:4, there were significant ($P < 0.05$) weak positive correlations between the length of shoots and E1S ($r = 0.086$) and PdG ($r = 0.214$). However, there was no significant correlation ($P > 0.05$) between the length of wheat shoots treated with dilution 1:14 and all measured reproductive hormones.

DISCUSSION

Seed germination test

No matter the type of seed, our control test results were consistent with the results of other authors who confirmed better seed germination rate in distilled water compared to urine-water solutions (Veena and Narendranath, 1993; Dilrukshi and Perera, 2009; Kubátová and Fedorova, 2016). We observed significant differences in the number of mung bean seeds that had germinated after 3 days, which is consistent with the results of previous studies (Narayana Swamy et al., 2010; Rine et al., 2014). The same authors (Narayana Swamy et al., 2010; Rine et al., 2014) measured the lengths of shoots on day 5 of a germination test, but according to our results, significant differences are readily visible after only 3 days, which is consistent with a study in female mithun (*Bos frontalis*) using paddy seeds (Perumal, 2014).

Our results from day 3 concerning germinated mung bean seeds and the lengths of their shoots are consistent with studies that reported an inhibitory effect of cows' pregnancy on mung bean germination (Dilrukshi and Perera, 2009; Rao Krishna and Veena, 2009; Skálová et al., 2017). Surprisingly, our results for wheat seeds germinating in the dilution 1:14 during days 1 and 2 correspond with those of Ghalioungui et al. (1963) when diagnosing pregnancy in humans. However, in cattle, the opposite trend would be expected (Veena and Narendranath, 1993), as we observed in mung beans. Nevertheless, with the exception of these two days in the 1:14 dilution, no differences were found in

either seed germination rate or shoot lengths of wheat seeds in the urine of pregnant and non-pregnant heifers. Thus, we can assume that the different varieties of wheat used might have different seedling emergence characteristics (Mohan *et al.*, 2013). Irrespective of the fact that seed germination rate is affected by different factors such as moisture, temperature, daylight, nutrition or seed storage and that different plant species have different requirements (White and Edwards, 2007), we applied 20 ml of urine-water solution to both kinds of seeds. While mung bean seeds almost depleted the whole solution due to significant swelling during their germination, wheat seeds were under the surface of the solution for the entire experimental period. In addition, we more frequently observed darkening of the seeds, colour changes in the urine-water solution or mildew in the Petri dishes that contained wheat seeds. It is possible that these factors influenced the results and precluded the finding of any differences at the end of the experiment.

Reproductive hormone assays

Urinary hormone levels are generally calibrated to creatinine values in urine (Kirkpatrick *et al.*, 1991; Yang *et al.*, 2003). However, normalizing against urinary creatinine was not needed for the purpose of this study because the exact concentrations of hormones were required to study their relationship with seed germination rate.

Our results for urinary E2 profiles in pregnant and non-pregnant heifers are consistent with studies carried out on E2 profiles in plasma (Robertson and King, 1979; Patel *et al.*, 1999). E2 concentrations increased as pregnancy advanced, and higher E2 concentrations were also confirmed in pregnant females.

Based on our results for urinary PdG as well as those from Yang *et al.* (2004), we consider urinary PdG monitoring to be a useful diagnostic method for pregnancy in heifers. Yang *et al.* (2004) reported three- to four-fold higher PdG concentrations in the urine of pregnant cows 21 days after AI. However, these results were obtained from only two

pregnant cows. Nevertheless, our results support these findings as they showed approximately eight times higher mean urinary PdG concentrations in heifers that were 4–8 weeks pregnant compared to non-pregnant heifers. Higher PdG concentrations in the urine of pregnant bison cows (*Bison bison*) were also reported by Kirkpatrick *et al.* (1991; 1992).

Interaction of reproductive urinary hormones and seed germination rate

Although Nirmala *et al.* (2008) determined that E2 and P4 are not efficient inhibiting/inducing factors of germination and growth, we found weak correlations between urinary reproductive hormone profiles and the germination and growth of seeds. Notably, we observed that urinary E2 and PdG correlated more frequently with seed germination rate and shoot growth than E1S. Moreover, the influence of E1S and PdG on seed germination rate has never been tested. We assume that mung beans germinated and grew less under the influence of higher urinary PdG concentrations.

Although some studies suggest that higher concentrations of ABA in the urine of pregnant female cattle result in seed dormancy by inhibiting germination (Veena Ganesaiah, 2006; Dilrukshi and Perera, 2009), our results demonstrate a different response of mung beans and wheat seeds to the urine of pregnant female heifers. Thus, we agree with the statement of Rao Krishna and Veena (2009) that "...plant growth regulators such as auxins and abscisic acid are likely to be excreted in urine as and when the animals consume plants containing such substances" and conclude that PdG is likely the main factor influencing germination, rather than ABA.

Our results confirm that the urine of pregnant heifers inhibits mung bean seeds germination and growth as evidenced by lower germination rates and shorter shoots. The most obvious effect on the germination of wheat seeds was observed in a 1:14 dilution

and displayed the opposite trend. Pregnancy status did not influence the length of wheat seed shoots. Higher concentrations of E1S, E2 and PdG were observed in the urine of pregnant heifers than in non-pregnant ones. We hypothesize that higher oestrogens and PdG concentrations in the urine of pregnant heifers can inhibit mung bean germination rate as well as shoot growth. Based on our results, we recommend mung beans rather than wheat seeds for the seed germination test because of better visibility of seedling emergence and clearer results compared with those obtained using wheat seeds. When sophisticated laboratory equipment or financial support is available, we recommend using hormonal assays for time efficiency, reliability, and consistency. Specifically, urinary PdG measurements can be regarded as a suitable method for early pregnancy diagnosis in heifers. Otherwise, a non-invasively processed seed germination test could serve as a urine-based alternative.

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5.5 Comparison of divergent breeding management strategies in two species of semi-captive eland in Senegal

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Scientific Reports

2020

Adopted from:

Kubátová A, Štochlová K, Brandlová K, Jůnková Vymyslická P, Černá Bolfíková B. 2020. Comparison of divergent breeding management strategies in two species of semi-captive eland in Senegal. Scientific Reports 10:8841(2020).

Authors' contributions:

For Supplementary material of the article, see Annex 3.

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Comparison of divergent breeding management strategies in two species of semi-captive eland in Senegal

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Breeding management of small populations may have a critical influence on the development of population characteristics in terms of levels of genetic diversity and inbreeding. Two populations of antelope sister species – Critically Endangered Western Derby eland (*Tauroragus derbianus derbianus*) and non-native Least Concern Cape eland (*Taurotragus oryx oryx*) bred under different management strategies were studied in Senegal, Western Africa. The aims of the study were to compare the population genetic parameters of the two species and to test for the presence of interspecific hybrids. In total, blood and tissue samples from 76 Western Derby elands and 26 Cape elands were investigated, using 12 microsatellite markers. No hybrid individuals were detected in the sampled animals within the multispecies enclosure in Bandia Reserve, Senegal. The parameters of genetic polymorphism indicated much lower genetic diversity in Western Derby elands compared to Cape elands. On the other hand, the coefficient of inbreeding was low in both species. It is hypothesized that this could be a positive effect of strict population management of Western Derby elands, which, despite the loss of genetic diversity, minimizes inbreeding.

Genetic diversity represents an essential pillar for the survival of populations through the possibility of adapting to a changing environment. Problems connected with maintaining diversity are common in captive populations, whose sizes are limited by space and the individuals are scattered among institutions worldwide so the gene flow is restricted[1–3].

To eliminate the negative impact of the phenomena affecting small populations (e.g. loss of variability due to genetic drift and inbreeding), it is necessary to apply appropriate genetic management[4,5], which can vary from intensive interventions[6,7] to simple monitoring[8]. However, for the decision-making process, it is essential to know some basic information about the kinship and genetic variability of the individuals[9] which is usually recorded in studbooks. Even if the studbooks for captive wildlife exist, for example, European studbooks[10] or International studbooks[11], knowledge of kinship across these populations is often limited and can be as low as only 50% or less in some ungulate species kept in zoos[12].

However, there are also species with better background information recorded, where a high proportion of their pedigree is known (% PK). These are mainly endangered species for which special *ex situ* conservation programmes have been created, such as the European Endangered Species Programmes (EEPs) for species like the Cuvier's gazelle (*Gazella cuvieri*, 100% PK)[13], or the recently established EAZA *Ex situ* Programmes[10].

Pedigree data are especially valuable in the evaluation of ancestry and kinship[14] but even the studbooks do not guarantee reliable information concerning genetic parameters of polymorphism within the populations. The reason for the lack of reliable genetic data is mostly because the studbook analyses rely on the assumption that the founders are unrelated and non-inbred, which is not always the case[6,15]. Only genetic monitoring can reveal the true genetic polymorphism of populations[16], including the ones without studbooks[8].

Loss of genetic variability is not the only issue of genetics in captive breeding of wildlife. The populations can face problems with interspecific hybridization, which is considered as a very important factor that endangers

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Characteristic/Population	WDE	CE
Origin of animals	Native Senegalese fauna	Introduced species
Conservation status	Critically Endangered	Least Concern
Population establishment	2000	1996
Source of the founders	Niokolo Koba National Park	South Africa
Number of founders	1 male: 5 females	3 males: 5 females
Population monitoring	Monitored (studbook publications, genetic monitoring)	No monitoring so far
Genetic management	Managed (1 bachelor and 5 reproductive herds, animal transports between herds, no culling)	Unmanaged (one herd, random males and recently even old diseased females culled for meat purposes)
Number of individuals	101 (June 2017, including the stock in Fathala Wildlife Reserve)	200–250 (February 2017, rough estimate of the Director for animals)
Resources	Brandlová et al.[22,26], Zemanová et al.[6], IUCN[45]	IUCN[46], Bandia Reserve veterinary records unpubl. data

Table 1. Overview of the background of the Western Derby eland (WDE) and Cape eland (CE) populations living in Bandia Reserve, Senegal.

biodiversity and the existence of many species[17]. Even if the hybridization occurs in nature, it can also be caused by human interference, usually by keeping related, but initially geographically isolated, taxa together; for example, in wildlife reserves or mixed-species exhibits in zoos, which is currently very popular[18–20].

Small isolated populations of two eland species with different conservation statuses and different geographical origins are kept in Bandia Reserve in Senegal. Both populations had a similar number of founding animals, but they have been managed differently and thus may serve as an ideal model for evaluation of the influence of population management on the genetic parameters of the population.

Western Derby elands (WDE, *Taurotragus derbianus derbianus*) are represented worldwide by less than 200 free-ranging individuals in Senegalese Niokolo Koba National Park (NKNP)[21]. A unique conservation programme for this antelope takes place in two Senegalese wildlife reserves, Bandia and Fathala, where about 100 of these antelopes live in semi-captive conditions[22]. When their founders were captured in NKNP in 2000, the total WDE population was already very limited and WDE was considered as Endangered. The population in NKNP was estimated to total only around 100 individuals at that time[23] and all the founders of the captive programme were captured from just one herd[24]. The founders were transported to Bandia Reserve where the first captive breeding of WDE started. In 2006, the primary selected animals from Bandia Reserve were transported to Fathala Wildlife Reserve[25].

According to the WDE Conservation Strategy, the conservation programme's "…aim is to manage the population to retain as high as possible genetic diversity…"[26]. For this purpose, the WDE semi-captive population is intensively managed to minimize kinship since its establishment[24] via annual identification of new-born calves and their mothers through suckling observations for studbook creation[27], transportation of sub-adult offspring to other breeding herds to avoid backcrossing[28], and quite recently even genetic monitoring to compare pedigree and microsatellite data[6]. However, natural gene flow between the reserves and the NKNP does not currently exist and the inbreeding rate of the population is increasing while the polymorphism is dropping rapidly[6]. On the other hand, Cape elands (CE, *Tauroragus oryx oryx*) were introduced to Bandia Reserve in 1996 to increase its attractiveness for safaris for visitors. The only management applied in the population of CE in Bandia Reserve involves the culling of surplus males and older calf-less females for meat production. Since the initiation of both breeding programmes, no animals have been imported into the populations of WDE and CE. For a comparison of the background of both studied populations, see the overview in Table 1.

In Bandia Reserve, all WDE were kept in fenced areas, separated from other species, until July 2012 when reserve managers decided to remove some of the fences in the reserve, and thus two previously separated breeding herds of WDE were merged and mixed with other animal species. Since that time, this WDE herd has been in physical contact with CE[26]. The length of pregnancy in the WDE is approximately 9 months[25], so all WDE offspring which have been born in the multispecies enclosure in Bandia Reserve since April 2013 should be considered as potential hybrids, in other words, 26 potential hybrids were born up from this period until June 2017[22].

Even though a hybrid of the Derby eland has never been observed, there is a risk of its hybridization with CE in Bandia Reserve, considering the previous experience with other Tragelaphini antelopes that are characterized by "unusual readiness to hybridize in captivity"[19,29–32]. Such hybridization would jeopardize the entire conservation programme and thus there is an urgent need for monitoring, as well as genetic variability assessment, and subsequent evaluation of the appropriateness of the applied population management.

The aims of this study were to:

1. Test for the presence of interspecific hybrids between semi-captive Western Derby elands (*T. derbianus derbianus*) and Cape elands (*T. oryx oryx*) living in a multispecies enclosure in Bandia Reserve, Senegal;
2. Compare the population genetic parameters of the two divergently managed species and evaluate the effect of the applied population management.

Materials and Methods

Sampling.   Individuals of two semi-captive populations were included in the study: WDE (n = 76) from Bandia and Fathala reserves in Senegal, and CE (n = 26) from Bandia Reserve, Senegal.

Blood and tissue samples of WDE were acquired systematically by continuous whole population sampling, and included all living individuals recorded in the studbook from 2017[22] with the exception of 25 animals, from which the samples were not available for various reasons (too young age, absence of veterinarian at the time of observation, etc.). Samples from CE were obtained by random sampling of live calves at the age of 1–3 years, animals that died naturally, and surplus males and older calf-less females culled for meat production by the staff of Bandia Reserve.

All samples were collected in Senegal in the period 2005–2017 by the NGO Derbianus Conservation (DC, formerly Derbianus Czech Society for African Wildlife) in cooperation with the Directorate of National Parks of Senegal (DPN) and Society for the Protection of Environment and Fauna in Senegal (SPEFS), who are recognized on an international level (Memorandum of Understanding between the Ministry of the Environment of the Czech Republic and the Ministry of the Environment of Senegal; Implementation Agreement to the Memorandum between the Ministry of the Environment of the Czech Republic, DC and the Czech University of Life Sciences Prague; Trilateral Agreement between DC, DPN, and SPEFS). The samples were then imported to the Czech Republic (requested veterinary conditions for import with reference numbers SVS/2210/2012-ÚVS, SVS/2015/007838-G and SVS/2017/032757-G have been fulfilled) and provided by the NGO for research purposes. No animal was sacrificed for this study. All sampling procedures were performed by certified veterinarians, except the collection of tissues from dead animals. Blood and hair samples were collected only from anesthetized WDE individuals during transport between breeding herds and the reserves, which regularly occurs because of the genetic management of the population[25]. Tissue samples were collected from both species either from dead individuals by scalpel or from living unanesthetized animals by Biopsy & DNA darts Pneu-Dart, Inc.

Immediately after collection, the hair and tissue samples were fixed in 96% ethanol, and the blood samples were treated with anticoagulants. Then, all the samples were transported to the freezers in the shortest possible time to be stored at −20 °C until processing in a laboratory which has approval (No CZ 11712934) for storage and usage of animal material according to § 48(1)(i) of Act No 166/1999s concerning veterinary care and amending certain related laws, as amended, pursuant to Article 17(1) of Regulation of the European Parliament and the Council (EC) No 169/2009 and Article 27(1) of Commission Regulation (EU) No 142/2011.

Processing of the samples.   Genomic DNA from hair and tissue samples was extracted by Qiagen® DNeasy® Blood & Tissue Kits. In the case of blood samples, Geneaid™ Genomic DNA Mini Kits (Blood-Cultured Cell) were used. Twelve microsatellite loci were selected for the analyses because microsatellites are fast evolving markers suitable for analyses of recent population structure[33].

The PCRs were carried out in T100™ Thermal Cyclers from Bio-Rad using the Qiagen® Multiplex PCR kit according to the enclosed protocol[34]. During reactions, 12 microsatellite loci were amplified with already published fluorescently labelled primers that were chosen based on cross-species amplification, including 5 previously used for WDE[6]; for details see Table S1 in Supplementary Material. Fragmentation analyses was performed using a 3500 Genetic Analyzer (Applied Biosystems™) with the GeneScan™ 500 LIZ™ dye Size Standard.

Data analysis.   Data from the fragmentation analyses containing information about allele lengths of individual loci were manually scored using GeneMarker® Version 2.2.0, SoftGenetics LLC®[35] and then binned by AutoBin[36]. Large allelic dropout, presence of stutter bands, and null alleles were tested in Micro-Checker 2.2[37]. Departures from the Hardy–Weinberg equilibrium and linkage equilibrium of all microsatellite loci were checked by Genepop 4.2[38].

Population structure and the presence of hybrids were tested by the Bayesian clustering method in Structure 2.3.4[39] with 1,000,000 steps of Markov Chain Monte Carlo repetitions after 100,000 steps of the burn-in period. The analysis was run for 1–5 clusters (K) always with 5 repetitions for each K. The results were post-processed by Structure Harvester to evaluate the best supported K[40].

Factorial correspondence analysis was done in Genetix 4.05[41]. To compare the populations of WDE and CE, basic population structure characteristics were obtained using different programs intended for population genetics calculations. Values of observed (Ho) and expected (He) heterozygosity were calculated in GenAlEx 6.502[42], inbreeding coefficient (F_{IS}), fixation index (F_{ST}) and allelic richness (Ar) were obtained via FSTAT 2.9.3.2[43], which was also used for the counting of the confidence interval of F_{ST}. The confidence interval of F_{IS} was evaluated using Genetix 4.05.

Results

In total, 76 samples of WDE and 26 samples of CE were genotyped. Analysis in Micro-Checker 2.2 did not detect any genotyping errors, including the presence of null alleles. Linkage disequilibrium was not detected. The populations were in Hardy–Weinberg equilibrium at all of the studied loci, except CE at ETH10 (p = 0.025) and WDE at CSRM42 (monomorphic locus in WDE).

The highest likelihood was obtained for two clusters (see Fig. S1 in Supplementary material). Each animal was correctly assigned to its presumptive species. The analysis did not reveal any signal of recent hybridization between the species (Fig. 1).

Factorial correspondence analysis visualized the relationships between all tested individuals of the tested populations (Fig. 2). Western Derby elands are represented by a homogenous cluster with little variation compared with CE.

Figure 1. Results from Structure showing assignment of the tested individuals (n = 102) into two clusters corresponding with their species – Western Derby eland (WDE, n = 76) and Cape eland (CE, n = 26).
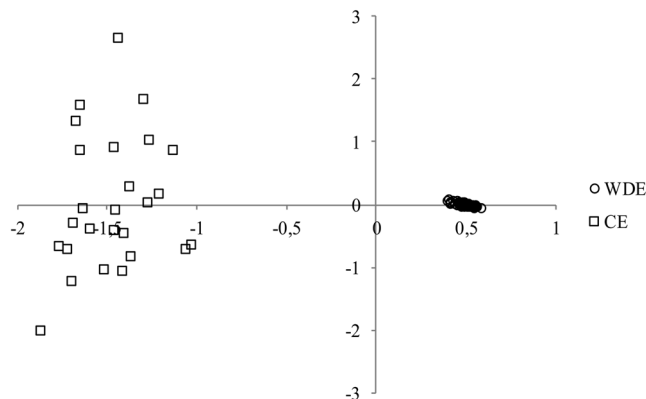

Figure 2. Results of the factorial correspondence analysis from Genetix based on 12 microsatellite loci showing a multivariate relationship between individuals of Western Derby elands (WDE, n = 76) and Cape elands (CE, n = 26).

Characteristic/Population	WDE (n = 76)	CE (n = 26)
He	0.425	0.755
Ho	0.445	0.771
Ar	2.462	5.873
Polymorphism	0.917	1.000
F_{IS} (95% confidence interval)	−0.046 (−0.061–0.003)	−0.021 (−0.096–0.003)
F_{ST} (95% confidence interval)	0.361 (0.295–0.429)	

Table 2. Values of basic population characteristics counted for both tested populations – Western Derby elands (WDE) and Cape elands (CE).

The results of the selected population structure characteristics to compare the populations of WDE and CE are presented in Table 2, and show higher heterozygosity and genetic diversity in CE than in WDE. However, both species have a low level of inbreeding.

Discussion

Derby elands (*Taurotragus derbianus*) and common elands (*Taurotragus oryx*) are considered as sympatric only in South Sudan[44–46]. So far, there is no information about the occurrence of hybridization between them, but it cannot be excluded, due to the high relatedness of the taxa (separation estimated at 1.6 million years before present)[47] and due to the presence of hybrids between other Tragelaphini species[19,29–32]. The present study did not detect any individual of hybrid origin. However, not all suspect individuals were sampled and thus some possible hybrids might have been overlooked. In the WDE population, 16 samples from totally 26 suspect animals were tested while three of them have died before being sampled and never reproduced. Moreover, samples were taken randomly in the CE population. It may be concluded that until June 2017, no hybrid was detected, and considering this, the probability of ongoing hybridization is low. However, the risk of hybridization between eland species in Bandia Reserve still exists, as the species remain in direct contact[48]. Also, one cannot exclude that post-zygotic reproduction isolating mechanisms may exist, and that the embryo may be lost during its development. Should this be the case, the fitness of the whole herd would decrease. Respecting the uniqueness and conservation status of the WDE, we propose continuous monitoring of hybridization between WDE and CE until they have separated breeding facilities. If the hybrids occur, it may have severe consequences for the genetic integrity of the species, as shown i.e. in the giant sable antelope (*Hippotragus niger variani*)[49] and bontebok (*Damaliscus pygargus pygargus*)[50].

Relatedness of the individuals and the sex ratio within the founding herd highly affected the genetic diversity of the studied populations. Parameters of genetic polymorphism (Ar, Ho) are much higher in CE (Table 2). Factorial correspondence analysis also supports higher variation within CE (Fig. 2). Two important factors should be considered: firstly, there is an assumption that the founders of the WDE semi-captive population were already related[6]. The second important factor to consider is the presence of just one male founder of the WDE semi-captive population[25], and thus the level of inbreeding within WDE increased rapidly over the generations[6]. In CE, the sex ratio of the founders was more balanced, containing three males and five females[51]. Although dominant eland males are usually considered as sires of all the offspring in their herds[25], this is not necessarily true considering the studies in other ungulate species[52,53].

Genetic management of the Critically Endangered WDE might positively affect the level of inbreeding of the whole population in semi-captivity. The values of the coefficient of inbreeding (Table 2) are comparable over the species, even though the other parameters of polymorphism are much lower in WDE (Table 2). Nevertheless, even though microsatellites were shown to be useful tools for the study of the genetic diversity of ungulates[54], they have their limitations[55], which must be considered regarding the results. They do not reflect the whole genome and therefore provides only partial information about the level of polymorphism, and often it is impossible to correlate specific traits with microsatellite parameters.

The genetic background of the population should always be considered from the onset of the establishment of a conservation programme. In optimal circumstances, there are more suitable candidate populations in the wild from which intended founders of the backup population can be selected. All founders should ideally be unrelated and their numbers sufficient to establish more breeding pairs to avoid kinship in the first generation[6,16,56]. However, this is not always possible in the case of Critically Endangered species, in which animal numbers have decreased to such an extent that backup breeding programmes must be established with only a few remaining founders[57]. An example of an extreme situation is the attempt to save the subspecies of northern white rhinoceros (*Ceratotherium simum cottoni*) via hybridization with the conspecific southern white rhinoceros (*Ceratotherium simum simum*), despite their natural long period of geographic, and thus genetic, isolation[58]. Nevertheless, there should be always an effort to avoid the possibility of both inbreeding and outbreeding depression, if possible[16].

Mitigating genetic threats of small isolated populations, such as inbreeding, and the negative consequences associated with the loss of genetic diversity can involve conservation actions such as translocations[8]. Even though such actions can be risky either from the epidemiological or genetic point of view[59], they may be crucial for species survival if managed properly[60]. In the case of the WDE semi-captive population, further importation of new individuals from NKNP to reduce inbreeding and increase genetic diversity has been already recommended[6,25]. The results of the present study regarding the genetic diversity of the WDE semi-captive population are in accordance with these previous conclusions and support the idea of introducing new founders from the wild. It also corresponds with the recommendations of Ochoa *et al.*[61] to promote mutual and continuous gene flow via translocations between populations in the wild and captive populations which should function as a source of genetic variation for reintroduction programmes. The One Plan Approach as an integrated approach to species conservation consisting of management strategies and conservation actions by all responsible parties for all populations of a species, whether inside or outside their natural range, should be a priority[62,63].

However, in the case of WDE, the promotion of gene flow is needed not only between the wild and captive populations, but also within the captive population. Otherwise, they could suffer from high differentiation as was described in the subpopulations of Arabian oryx[61]. Although the WDE semi-captive population was considered as a whole in the present study, the individuals are kept in two separate reserves, and regular animal transport takes place between the breeding herds, mostly within each reserve. The transport of animals from Bandia Reserve to Fathala Wildlife Reserve took place in 2006, 2008, 2009 and 2011, with a total of 32 individuals (22 males and 10 females) being translocated and organized into one bachelor herd at first, and later, two breeding herds[25]. Since only a limited number of females succeeded in reproducing in Fathala Wildlife Reserve, the population in Fathala suffered multiple founder effects, and pedigree analysis suggests considerably lower genetic diversity in Fathala Wildlife Reserve than in Bandia Reserve[1–3,64].

Wespi *et al.*[7] concluded that population management including interventions possibly influencing genetic diversity via regular changes of breeding males does not always offer distinct advantages when compared to unmanaged populations. However, their study did not reflect genetic parameters. In contrast, the results of the present study indicates that genetic management could have a positive effect on the genetic background of populations. Considering the results of this and a previous study by the present authors[6], it may be concluded, that for highly inbred populations such as WDE, genetic management keeps inbreeding at a low level, despite low genetic polymorphism and high relatedness of the population.

Data availability

Upon acceptance of the manuscript, genotypes used in the final analyses will be deposited at Dryad Digital Repository.

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Author contributions

Study was designed by K.B. and B.Č.B., samples were collected by K.B., P.J.V., A.K. and K.Š., samples were processed by A.K. and K.Š., data were analysed by B.Č.B., A.K. and K.Š., manuscript was drafted by A.K. and all authors contributed to the final version, financial support was provided by K.B., B.Č.B. and P.J.V.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-65598-6.

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6 Discussion

The results of the thesis are comprised of articles focused on variable topics in more species. However, all of them are connected and related to the topic of the thesis – genetic monitoring and reproduction management of captive ungulate populations.

Largely the thesis is focused on the pregnancy diagnosis as an important part of reproduction management. The methods which were used have in common their non-invasiveness and special attention to diagnosis from urine. The pregnancy diagnosis should be reliable enough, user-friendly, examinant-, animal-, and concept-friendly (Gargiulo et al. 2012). It should also be simple (Krishna Rao & Veena 2009), economical, and time undemanding (Lalrintluanga & Dutta 2009). Ultrasound fulfils all these requirements. It can be a very fast method when it is done by an experienced sonographer (Lamb & Fricke 2005) and can be even used as a non-invasive method (Kähn et al. 1993; Hunnam et al. 2009; Aziz & Lazim 2012). Ultrasound is often used both in domestic animals (Kähn et al. 1993; Lamb & Fricke 2005; Hunnam et al. 2009) and wildlife (McNay 2006; Kleiman et al. 2010).

However, in some cases, the ultrasound may be too expensive to get or keep, too complicated to use, or just inaccessible (Ndu et al. 2000a; Ndu et al. 2000b). In these situations, other methods of pregnancy diagnosis are needed. They should be (besides requirements mentioned above) cheap, affordable (Ndu et al. 2000a; Ndu et al. 2000b), farmer-friendly, non-invasive (Dilrukshi & Perera 2009), and requiring neither restraining of animals nor specialists for interpretation of the results (Rao Krishna & Veena 2009). Ideal methods for both domestic animals and wildlife should be mainly door step techniques that can be used directly in place with the help of cheap materials and with no requirements of special skills (Narayana Swamy et al. 2010). Therefore, simple and accessible methods already verified as pregnancy diagnostic tests in other species have been tested as a part of this dissertation thesis.

The results of the article entitled "Urinalysis of pregnant and non-pregnant alpacas (*Vicugna pacos*) and Bactrian camels (*Camelus bactrianus*)" did not support previously published results about the usage of urinalysis for pregnancy diagnosis in camels (Banerjee et al. 1981). No connection between the urinalysis results and the pregnancy status was found even in alpacas. Moreover, the results suggest that the urinalysis is not

applicable as a part of pregnancy diagnosis even in other species of camelids. The results can save time to those breeders who would like to try to use this simple analysis for the purpose of pregnancy diagnosis in camelids.

The second article "Non-invasive pregnancy diagnosis from urine by the Cuboni reaction and the barium chloride test in donkeys (*Equus asinus*) and alpacas (*Vicugna pacos*)" concluded that the Cuboni reaction seems applicable for pregnancy diagnosis in jennies. The Cuboni reaction has many advantages: a collection of urine can be done by breeders themselves. The reaction can be carried out by accredited laboratories and the price is acceptable. Therefore, the breeders can manage the pregnancy diagnosis intheir jennies without the need for veterinary assistance. Because of this, the Cuboni reaction has the potential to become a standard pregnancy diagnostic method in donkeys. Based on these results, the Cuboni reaction could also be tested in other equid species, e.g. endangered species of zebras, wild asses, or Przewalski's horses.

The results presented in the article "Seed germination test as an alternative urine-based non-invasive pregnancy test in alpacas (*Vicugna pacos*)" suggest that the seed germination test with mung beans seems to be a viable method for pregnancy diagnosis in alpacas, but further research is needed. The problematics of the seed germination test has been further studied and described in the article "Urinary reproductive hormones influence seed germination within diluted urine of heifers" which recommended mung beans rather than wheat seeds for the seed germination test because of better visibility of seedling emergence and clearer results compared with those obtained using wheat seeds. This conclusion can facilitate the usage of the test and readability of the obtained results. Moreover, one part of this study investigated the influence of urinary reproductive hormones (oestrone sulphate, 17β-oestradiol, and pregnanediol-3-glucuronide) on the germination of seeds kept in diluted urine of cattle, and the results suggested that urinary reproductive hormones could influence seed germination and growth. This is in contrast with the only one study examining the effects of different concentrations of oestrogen and progesterone on mung beans and wheat seeds, which concluded that they do not influence seed germination (Nirmala et al. 2008).

The last article focuses on another important part of population management – genetic management, which should always go hand in hand with the reproduction management. The results presented in the last article "Comparison of divergent breeding management strategies in two species of semi-captive eland in Senegal" provide new

information concerning population structure and characteristics of the Western Derby elands kept in semi-captive conditions in Bandia and Fathala reserves in Senegal. These results are usable in the evaluation of the appropriateness of the applied population management within the Western Derby Eland Conservation Programme, but could be applied even in other species and their conservation programmes.

7 Conclusions

All the journal articles included as the results of this thesis present new findings in the areas of reproduction and genetic management of specific odd-toed and even-toed ungulate species. Within this thesis, the Cuboni reaction has been verified in jennies as a simple, non-invasive, and affordable method for pregnancy diagnosis. The results of the thesis also suggest that hormonal levels should be a priority when looking for factors causing seed germination inhibition during seed germination testing as a pregnancy diagnostic method. Further studies in this area could reveal other species as candidates in which this test could be applicable. The same can be recommended in the case of the barium chloride test. Negative results obtained in tested species indicate that further knowledge of the factor(s) preventing and inducing precipitation should be a priority in this area. Considering genetic management, the results of this thesis may conclude that in highly inbred ungulate populations, genetic management keeps inbreeding at a low level in spite of low genetic polymorphism and high relatedness of the population. Therefore, in similar cases it is possible to recommend genetic management based on relevant data in comparison with the application of no population management at all.

As it was previously stated, obtained results can contribute to better population management of the examined species. Moreover, tested species can be used as model animals for other endangered species under conservation programmes. In the case of tested domestic animals, the results could be applied to a range of domestic breeds including the endangered ones facing possible extinction.

Detailed recommendations for further research pertaining to obtained results are described in individual articles. Nevertheless, not only future research but also the usage of currently known facts in practice is very important. Therefore, all the results have been consulted with breeders and other stakeholders who agreed to cooperate and participate in the research connected with this dissertation thesis. Many thanks belong to them.

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Annexes

List of Annexes:

Annex 1:   Author's CV

Annex 2:   Author's publications

Annex 3:   Supplementary material of the article from the section 5.5

Annex 1: Author's CV

Education:

2016 – present	**Doctoral Programme Tropical Agrobiology and Bioresource Management**, CZU, FTA. Thesis title: Genetic Monitoring and Reproduction Management of Captive Ungulate Populations.
2014 – 2016	Doctoral Programme Agriculture in Tropics and Subtropics, CZU, FTA. Ended due to change of study programme.
2013 – 2015	**Master's Programme Exotic Animals Breeding**; CZU; FAFNR. Thesis title: Reproductive Status Monitoring by Saliva Crystallization in Non-human Great Apes.
2012 – 2014	**Master's Programme Animal and Food Science in Tropics and Subtropics**, CZU, FTA. Thesis title: Pregnancy Diagnosis from Urine in Even-toed Ungulates.
2009 – 2012	**Bachelor's Programme Tropical and Subtropical Agriculture**, CZU, ITS. Thesis title: Research in Zoological Gardens – Problems, Trends, and Perspectives.

Work experience:

2017/07 – present	**Wildlife inspector**, Czech Environmental Inspectorate, Unit of International Protection of Biodiversity and CITES.
2017/01 – 2018/12	**Main coordinator of project** "Small populations of endangered antelope species, risks of their extinction and possibilities of prevention" financed by Internal Grant Agency of CZU.
2016/04 – 2017/03	**Technician**, CZU, FTA, Department of Animal Science and Food Processing.
2014/07 – 2016/03	**Administrative officer** for international relations with Turkey and Azerbaijan at the Ministry of Agriculture of the Czech Republic.

Language skills:

Czech	Native speaker.
English	B2 exam.
Spanish	B1 exam.
Slovak	Passive knowledge.
German	Elementary passive knowledge.

Other skills and experience:

Internships abroad

2016, 2017	Two one-month stays in Senegal connected to the PhD. studies.
2011/08 – 2011/09	One-month study stay in Vietnam.

Volunteering

2012/12 – 2018/12	Derbianus Conservation z. s. (former Derbianus Czech Society for African Wildlife), since 2016/04 member of Executive Committee.
2012/09 – 2014/01	Greenpeace Czech Republic, Ocean Campaign, Tuna Guide 2014.
2005/08 – 2008/01	Volunteer centre Lékořice in Thomayer Hospital, Prague.

PC skills	MS Office, EndNote, Statistica, GeneMarker, Genetix, Genepop, GenAlEx, Structure.

Annex 2: Author's publications

Scientific articles:

Kubátová A, Štochlová K, Brandlová K, Jůnková Vymyslická P, Černá Bolfíková B. 2020. Comparison of divergent breeding management strategies in two species of semi-captive eland in Senegal, Senegal. Scientific Reports 10: 8841(2020).

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Proceedings papers:

Zelený J, **Kubátová A**. 2016. Párování steaků z mas exotických druhů kopytníků s víny z odrůd révy vinné pěstovaných v českých a moravských vinařských regionech. 8th International Annual Scientific Conference on Hotel Services, Tourism and Education. Prague.

Abstracts of author's oral presentations at conferences:

Kubátová A, Štochlová K, Brandlová K, Černá Bolfíková B. 2017. Western Derby Eland – Small Population with Big Aims. Page 49. Antelope, Giraffe, Hippo in the 21st Century: Conservation Action in Africa. Czech University of Life Sciences Prague, Prague.

Kubátová A, Štochlová K, Brandlová K, Černá Bolfíková B. 2017. Malá populace s velkými cíli: záchranný program pro západní antilopu Derbyho. Page 113. Zoologické dny. Brno.

Selected abstracts of author's posters presented at conferences:

Štochlová K, **Kubátová A**, Brandlová K, Černá Bolfíková B. 2017. Phylogeny and population characteristics of Derby eland (*Taurotragus derbianus*). Antelope, Giraffe, Hippo in the 21st Century: Conservation Action in Africa. Czech University of Life Sciences Prague, Prague.

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Annex 3: Supplementary material of the article from the section 5.5

Table S1: List of 12 microsatellite primers in the form of three primer mixes that were used in the study with the annealing temperature 58 °C:

primer mix 1	BL42 [64], BRR [65], CSRM60 [65], ETH10 [65], ETH225 [6], X800214 [6]
primer mix 2	BM4505 [6], CSSM42 [66], INRA107 [66], SPS113 [66]
primer mix 3	AF533518 [6], OarFCB304 [6]

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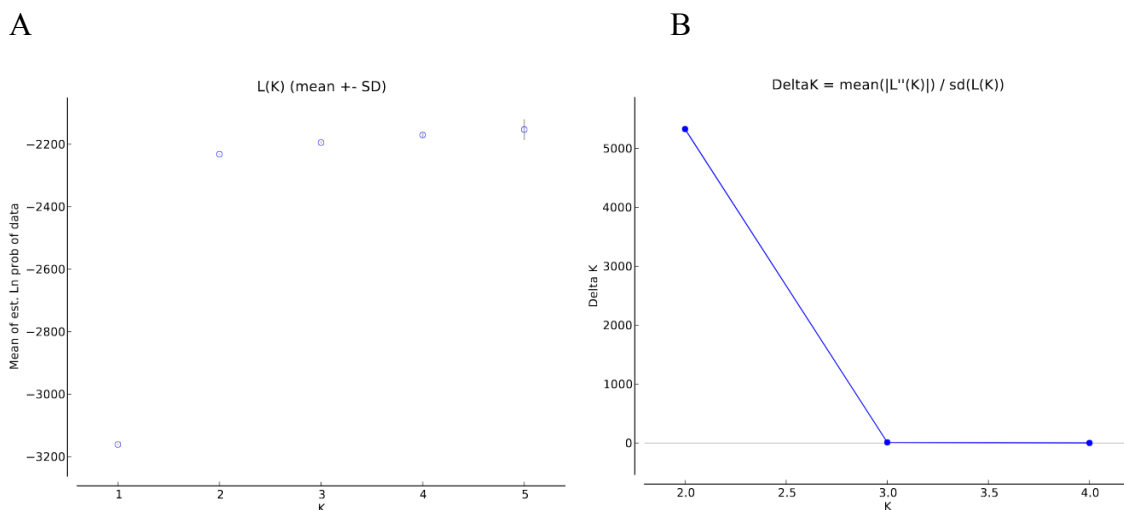
A   B

Fig. S1: Plots generated from Structure Harvester. A: Mean likelihood L(K) and variance per K value from Structure on a dataset containing 102 individuals genotyped for 12 microsatellite loci. B: Evanno's delta K statistic plot detecting the number of K groups that best fit the data, here 2 clusters.