Vaginal swab cytology aplication to determine the estrus cycle of lowland anoa (*bubalus depressicornis,* smith, 1927) in captivity

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Abstract. Anoa is an endemic species of Indonesia that is listed as endangered in the IUCN Redlist list and also included in the Appendix I CITES. Anoa's reproduction have been constraint due to their natural behaviour. This animal is a solitary animal, monogamous, wild and aggressive, Incorrect mating time can lead the into a fight between the male and female. Information about the estrus cycle is very important in determining the optimal mating time for anoa. The study aim is to determine the estrus cycle of anoa based on behaviour and change in the vaginal epithelial cells. Behavioural data were observed by focal animal sampling technique to 3 female anoas in Anoa Breeding Center Manado during January-August 2017 at 07.00-17.00 CIT. Vaginal swabs cytology was done during July-August 2017 in the morning and afternoon by using terilized cotton swab and Giemsa stain. The vaginal swabs cytology techniques with Giemsa staining can be used to determine the changes in epithelial cells and confirm the estrus phase of anoa. Based on vaginal cytology the length of the estrus cycle of each individual anoa at the Anoa Breeding Center is different. At Manis the lengt of estrus cycle ranges 23 days, Rita 15 days, Denok 21 days and Ana is unidentified

1 Introduction

Anoa is an endemic species of Sulawesi and Buton Island. There are two species of it, they are mountain anoa (Bubalus quarlesi) and lowland anoa (Bubalus depressicornis) [1-4]. Anoa is considered as endangered species [4-6], listed on appendix I based on the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and fully protected under Indonesian law. The major threats of the population in nature are hunting and habitat loss [2,6]. The rate of population decline is estimated at 14-18% per year [3].

Anoa is a solitary animal, wild and monogamous, thus affecting the growth of the population in nature and even affect the success of captive breeding [2]. Anoa is included in the polyestrus animal and at the time of estrus they will release pheromones that can stimulate the males to reach the females [7]. Anoa will be more aggressive during the lust [8]. When a male anoa meets a female, there will be a fight so the process of mating will be difficult to be happen [9]. The female Anoa will be willing to accept the male while experiencing the peak of the estrus that occurred only a few hours. Information on the estrus cycle is very important as a basis for determining the proper time of natural mating. The reserach on the estrus cycle of anoa shows varying results, i.e 26 and 30 days [8], 21 days [10], 16-31 days and 22-23 days [12]

The vaginal cytology is can be used to diagnose the stage of estrus cycle in many species [12-14), it reflects the interaction of various hormones on the reproductive tract [13]. Vaginal smear is indicating an increase in cornified acidophilic cells during the estrus of buffalo [15]. The study aim is to determining the estrus cycle and duration of estrus periode in anoa at Anoa Breeding Centre through cytologyc observation of vaginal epithelial cell.

2 Material and methods

2.1 Study area

The research was conducted at Anoa Breeding Center Manado which is located in the office of Environment

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and Forestry Research and Development Institute of Manado, Ministry of Environment and Forestry. The study was conducted on January to September 2017

2.2. Materials

Four female Anoa beetween 3,5 years old and 8 years old were used in the study (Fig 1). Sterile cotton swab (2,2 mm x 150 mm, Giemsa stain, glass side, and NaCL 0,9% were used for cell epetheal collection, and michroschope were used for examined the cell in the glass slide.

Remark : a. Anoa "Rita" 3,5 y.o; b. Anoa "Denok" 7,5 y., c. Anoa "Ana" 6 y.o dan d. Anoa "Manis" 8 y.o

Fig. 1. Female anoa at Anoa Breeding Centre Manado.

2.3. Procedure

Behavioural observation were conducted from each animal during January and August 2017 and vaginal swabs during July and September 2017. The anoa were placed in the reproductive enclosure. The behavioural changes observed by focal animal sampling technique. The reviews of epihelial cells observed by vaginal swabs cytology. Behavioural estrus were compared to the vaginal cytology obtained.

Sterile cotton swab (2,2 mm x 150 mm) were used for cell collection. Cotton swab were moistened with NaCL 0,9% and introduced into the vagina approximately 1,5 cm, quickly and gently rotated against the floor of the vagina. The epithelial cells were obtained immediately and being prepared by rotating the cotton swab along a glass side. The smear was fixed using bunsen burner. Then stained with Giemsa and leave it for 10-20 minutes. Then washed it in the tap water, leave it air dried for a moment and fixed again using bunsen burner. The stained slides were examined using michroschope with magnification of 40X. The classification of the cells can be seen on Table 1.

Table 1. The characteristic of epithelial cells.

Estrus phase	The dominant cells	Cell shape
Diestrus	Parabasal cells	Small cell, round with a large nucleus
Diestrus – proestrus	Intermediet cells	A round cell, larger than a parabasal cell with a smaller nucleus
Proestrus – estrus	Intermediet / superfisial cells	Large cell, polygonal shape, nucleus is very small or no nucleus
Estrus	Superfisial conificated cells	Large cell, polygonal shape, nucleus is very small or no nucleus and keratinization occur

2.4. Data analysis

Data is processed descriptively and presented in tables, pictures and graphs. The collected vaginal samples were then identified based on the predominantly emerging cells as Judi [11] which classified them into 4 phases. Ther are Estrus, Metaestrus, Diestrus and Proestrus with cellular characteristics in each phase referring to Najamudin [13].

3 Result and discussion

3.1. Result

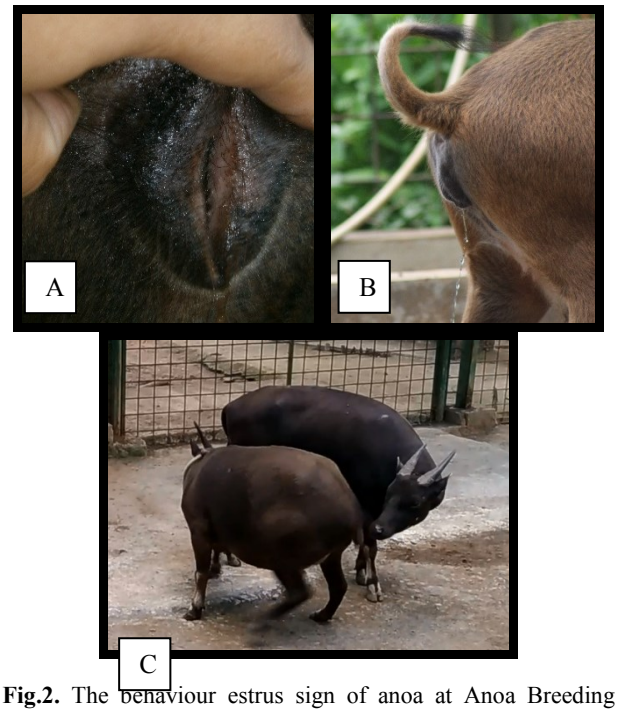
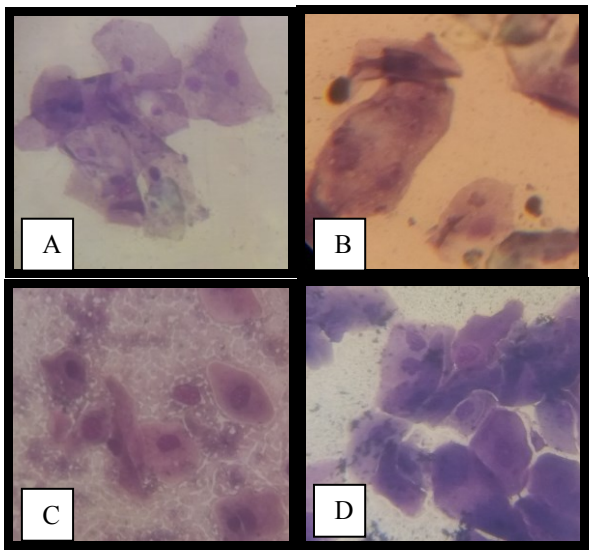


Fig.2. The behaviour estrus sign of anoa at Anoa Breeding Centre.

Based on Fig. 3 there are several signs of estrus found in the anoa in ABC that changes in the vulva (red, swollen, wet) and the release of cervical mucus. Usually a female anoa will lift and wag their tails. This change encourages the male anoa to approach, follow and smell the genital part of the female anoa.

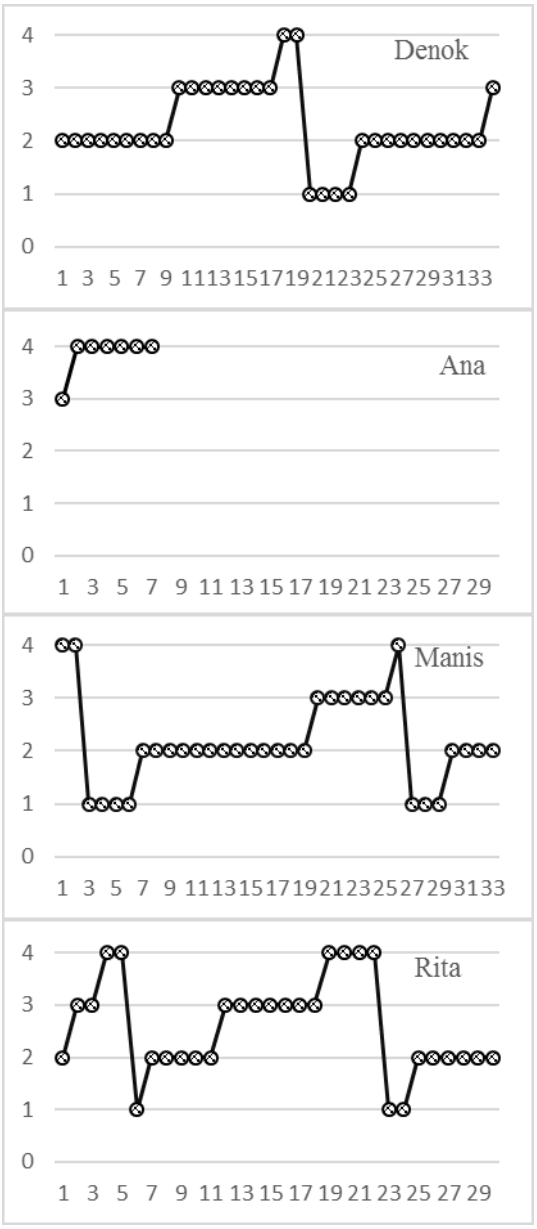


Remark : A) intermediet cells dominant (Proestrus), B) superfisial cells dominant (Estrus), C) parabasal cells dominant (Diestrus), D) superfisial dan parabasal cells dominant (Metestrus)

Fig. 3. The epithelial cells features of anoa in captivity.

Anoa vaginal epithelial cells are divided into 3 types of cells. There are parabasal, intermediate, and superficial [12]. Based on Fig. 3 proestrus phases are dominated by intermediate cells, polygonal in shape, 2-3x larger than parabasal cells, the ratio of nuclei to cytoplasm is small. The estrus phase is dominated by superficial cells, polygonal, flat or folded appearance. In this phase the epithelial cells begin to lose the core (cornified). The diestrus phase is dominated by parabasal cells, almost spherical, the ratio of nuclei to the cytoplasm is large. The metestrus phase is characterized by a balanced number of parabasal and superficial cells.

Based on Fig. 4 elow note that the four anoas have different estrus length cycles. Manis estrus cycles are recorded 2 times with a 23 day cycle length and a duration of 1-2 days. The length of the Rita estrus cycle is 15 days with a duration of 2-4 days estrus. We could not determined the estrus cycle of Ana because of its epithelial cell observation is always dominated by parabasal cells (see also Fig. 5)



(Y axis (1. Metaestrus, 2. Diestrus, 3. Proestrus, 4. Estrus) X axis. Observation day at-)

Fig. 4. The estrus phase observation.

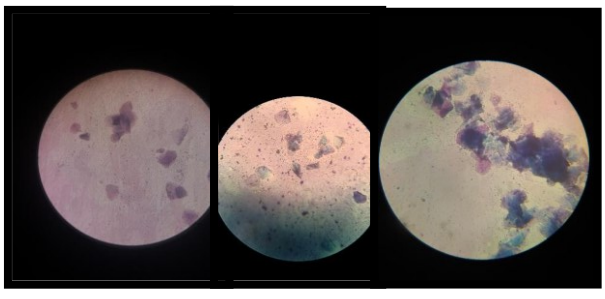


Fig. 5. Fig. 5. Ana epithelial cell features on 13, 11 and 16 August 2017

3.1 Discussion

The vaginal cytology is performed to observe the type and proportion of each epithelial cell. The vagina smear

shows a change in the dominance pattern of epithelial cell types. The dominance of epithelial cell types describes different estrus cycles. In this research we classified the epithelial cells into one of three fundamental types, parabasal, intermediate or superficial cell. It is classified into three cell types according to the size differences as a superficial squamous cells (40-65um diameter) with light cytoplasm, intermediate squamous cells (20-40um diameter) and parabasal cells (12-15um diameter) [13].

Based on the result (Fig.3), there are 3 types of epithelial cells. The diestrus phase is dominated by parabasal cells, proestrus phase is dominated by intermediate cell, estrus phase is dominated by cornified superfisial cell, and metestrus phase is dominated by superfisial and parabasal cell [11]. The parabasal cell is the smallest epithelial cell. It can be found when smears taken during diestrus, uncommon during early proestrus, absent during estrus. *Bubalus quarlesi* the diestrus phase occurs between 5-11 days [7]. Intermediate cells vary in shape and size (small and large intermediate). It is can be found in all stages except estrus. It is typically have diameter two to three timer larger than parabasal cell [najamudin]. The superficial cell is the largest epithelial cell seen in vaginal smear. It is polygonal in shape and distinctly flat, sometimes folded [11]. Its nuclei is either absent or pyknotic (very small and dark). Superficial cells without nuclei are often referred to as being fully cornified. In the estrus phase there are many cornified epithelial cells as a result of high estrogen levels [Sharman]. Based on result the dominance of superficial cells can be observed for 1-2 days during the estrus phase. When female anoa in estrus its vulva become swollen, reddish, and wet, as well as out cervical mucus [Fig 2]. This mucus serves to reduce irritation when copulation occurs. The male anoa will follow, kiss and lick the genital parts of estrus female (Fig.3). The next phase is metestrus. In this phase we will find superficial and parabased cells appearing in equal proportions.

Previously Denok undergoes postpartum anestrus for 6 months because of breastfeeding activity. Denok was never shown the estrus sign during the breastfeeding. As happen in the mud buffalo (*Bubalus bubalis*) which ovulate on the 39th day after the birth [16]. Births will have an effect on ovarian activity and follicular development causing absence of ovulation, 53% of cows show anestrus conditionafter birth until the next mating season [15]. It was difficult to take vaginal smear of Denok because she was very aggresif during the breastfeeding periode. Vaginal smear on Denok done when the calf is weaned and she was tranfered to breeding enclosure along with other anoa. The result shows that Denok estrus cycle length is 21 days. The results of vaginal swab observations on Ana could not be identified / inferred because of doubt about the image of the cell type shown. Ana epithelial cells are always in a superficial condition (Fig.5). Normally dominated superficial cell only present in estrus phase whic is only one to two days periode. Manis estrus were observed two times with an estrus cycle ranging at 23 days and duration 1-2 days. During the estrus phase, when Manis and Rambo were mated, the appetite were decreased, their food were still plenty left till afternoon. Based on vaginal swab review Rita estrus cycle ranging at 15 days with the periode of estrus 2-4 days. The results is very different, compared to the Denok and Manis, Rita have the shortest estrus cycle.

4 Conclusions

The vaginal swabs cytology techniques with Giemsa staining is can be used to determine the changes in epithelial cells. It can be used to confirm the phase of estrus phase of anoa. Based on vaginal cytology, the length of the estrus cycle of each individual anoa at the Anoa Breeding Center is different. At Manis the lengt of estrus cycle ranges 23 days, 15 days for Rita, 21 days for Denok and unidentified for Ana.

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