Non-invasive measurement of cortisol metabolites in feces as an indicator of stress and its relationship with the number and arrival frequency of visitors in captive sambar deer (Cervus unicolor)

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Abstract. Nowadays, the non-invasive measurement of cortisol in feces is a popular method used as an indicator of stress in wild and captive animals. This study was conducted to examine the feasibility of a non-invasive method for cortisol metabolites measurements in feces and investigate its relationship with the number and arrival frequency of visitors in captive Sambar deer. In total 64 fecal samples were collected together with the observation of the number and arrival frequency of visitors from 7 Sambar deers (3 adult males, 4 adult females) rearing in zoos of Taman Rusa Lamtanjong, Aceh Besar, Indonesia. Subsequently, fecal samples were extracted and the concentration of cortisol was measured by using 3α,11β-dihydroxy-etiocholanolone assay. Data were analyzed using a t-test and Pearson correlation. Results showed that cortisol metabolites concentration in adult males of Sambar deer (276.20±52.74 ng/g dry feces) was higher compared to adult females (181.56±25.87 ng/g dry feces). The concentration of cortisol metabolites was significantly correlated with the number of visitors (r = 0.482, p < 0.05) and the arrival frequency of visitors (r = 0.398, p < 0.05) in which the higher number and arrival frequency of visitors increased the cortisol metabolites concentration. In conclusion, the concentration of cortisol metabolites in Sambar deer can be measured non-invasively from feces and associated with the number and arrival frequency of visitors.

1. Introduction
Sambar deer (Cervus unicolor) is one of the three native deer in Indonesia namely Timor deer (Cervus timorensis) and Bawean deer (Axis kuhlii) [1]. Sambar deer have a large body (adult females: 130-150 kg, adult males: 200-250 kg), have secondary sexual characteristics in the form of antler in males and have an attractive color [2,3]. Therefore, these animals have good potential to be maintained as a tourist attraction (ecotourism) as well as an alternative source of meat and antler. In 2006, the population of

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Sambar deer in its habitat was not too worrying and categorized as low risk, but since 2008, the status of this animal has increased to become vulnerable, due to population decline reaching 50% [4]. This occurs due to illegal poaching, forest (habitat) destruction and limited reproductive capacity of Sambar deer. According to a decision letter of Minister of Forestry No. 305 / Kpts-11/1991, 19 June 1991 and Government Regulation (PP) No. 7/1999, Sambar deer have been registered as a protected species.

In order to increase the population of Sambar deer, ex-situ conservation has been carried out. Ex-situ conservation is an effort to rearing and breeding a species outside of their natural habitat such as in zoos. Unfortunately, apart from being a conservation effort, in zoos, Sambar deer is also used as object of ecotourism activities. This activity creates an unavoidable interaction between deer and humans (visitors) which is feared that it will cause stress [5]. According to Choo et al. [6], several variables can cause stress to animals in zoos including the number of visitors and visitor activities. The density (number) of visitors in the ecotourism area has been reported to be positively correlated with the increase of aggressive behavior in animals [7,8]. In addition, visitor activities such as talking too loudly (noisily) and close interaction of visitors with animals can cause stress in animals [9]. One of the indicators that animals have experienced stress is an increase in abnormal behavior (stereotype behavior), increase aggressive behavior among animals, or even attacks on visitors [10]. Thus, it may affect animal welfare. Therefore, it is important to monitor stress levels in this animal by measurement of stress hormones such as cortisol, because it is a good indicator of stress [11].

In the last few decades, a non-invasive technique for the measurement of stress hormones from fecal samples has been developed [12]. This technique is suitable for Sambar deer who are prone to stress. The advantages of the non-invasive method over an invasive method are 1) sample collection is easier and can be done over long periods, 2) does not disturb and endanger animals due to the restraint or anesthesia, 3) animals do not experience stress due to wrong handling and 4) measured stress hormone concentrations are more valid [13]. Non-invasive measurement of the stress hormone has been successfully applied to various taxa such as primates [14], herbivores [15], carnivores [16], birds and poultry [17], aquatic mammals [18], reptiles and amphibians [19]. This method is possible to be used because feces contain the metabolized forms of all steroid hormones such as progesterone, estrogen [20], testosterone and glucocorticoids (cortisol metabolites) [12]. The present study aims to examine the feasibility of a non-invasive method for cortisol metabolites measurements in feces of Sambar deer using the 3α,11β-dihydroxy-etiocholanolone assay. Furthermore, investigate the relationship between the number and arrival frequency of visitors on the cortisol metabolite concentration of Sambar deer in captivity.

2. Materials and methods

2.1. Study site and animals
The study was conducted in the zoo of Taman Rusa Lamtanjong, Aceh Besar, Indonesia for two months. In total, 64 fecal samples were collected from seven Sambar deer consisted of three adult males (4-5 years old) and four adult females (3-4 years old). Sample collection was performed in the morning (08.00 to 10.00 a.m).

2.2. Fecal sample collection
Fresh fecal samples were collected immediately after defecation. Before putting it into a sample tube, fecal samples were homogenized using a stick. Approximately, 20 g of homogenized fecal sample was then put into a fecal tube and stored in a cooler box. After that, the fecal sample was transported to the Physiology Laboratory, Faculty of Veterinary Medicine, Universitas Syiah Kuala and stored at -20°C in the freezer before extracted and measured for cortisol metabolites measurement.
2.3. Observation of number and arrival frequency of visitors
Observation of number and arrival frequency of visitors was carried out from morning (08.00 a.m) till evening (05.00 p.m). The number and arrival frequency of visitors were calculated based on the visitors that visited the Sambar deer cage per day.

2.4. Fecal extraction
The procedure of fecal extraction was adopted from the method of Gholib et al.,[14,15]. The first, samples were taken out from the freezer and then thawed at 50°C for 1 to 2 hours. Approximately 0.5-0.6 g sample was put into a 15 ml centrifuge tube containing 4.5 ml of 80% methanol. The sample was then extracted by using a multivortexer at 1000 rpm for 15 minutes. After that, the fecal solution was then centrifuged at 3000 rpm for 10 minutes. Finally, the fecal extract was taken and filled into a 1.5 ml microtube and stored at -20°C in the freezer before cortisol metabolites measurement.

2.5. Hormone analysis
The concentrations of cortisol metabolites were measured by using a competitive enzyme immunoassay using a specific antibody "5β-androstane 3α,11β-diol-17-CMO-BSA (11β-hydroxy etiocholanolone)". Fecal extract of Sambar deer has measured the cortisol metabolite concentration as described by Gholib et al. [14,15,21]. First, 50 µl of fecal extract, the standard of 11β-hydroxy etiocholanolone and quality control were taken using a micropipette and filled into a microplate. Second, 50 µl enzyme conjugate and 50µl antibody of 11β-hydroxy etiocholanolone were added into each well of microplate and then mixed. The microplate was then incubated for 12 to 16 hours at 4°C in a refrigerator. After incubation, the microplate was washed with washing buffer four times and then blotted dry using a towel paper. After that, each well of the microplate was added by 150 µl of streptavidin-peroxidase (Sigma, Germany) and incubated at room temperature for 30 minutes in dark. Furthermore, the microplate was washed four times using washing solution. After washing, each well of the microplate was added with 150 µl substrate solution and then incubated for 30-45 minutes in dark. Each well of microplate was added 50 µl stop solution (2 M H2SO4) to stop the enzyme reaction. Finally, absorbance was measured using the microplate spectrophotometer (ELISA reader, Bio-Rad Laboratories Inc.) at 450 nm and the cortisol metabolite concentration was calculated by using a Program of MPM 6.

2.6. Data analysis
Data of cortisol metabolites concentration from adult males and adult females were analyzed by using Student's t-test. The correlation between the number and arrival frequency of visitors and the cortisol metabolite concentration was analyzed by using the Person's correlation test. Data analyses were performed by using SPSS 20 and statistical significance was set to α = 0.05.

3. Results and discussion

3.1. The concentration of cortisol metabolites in adult males and females of Sambar deer
The cortisol metabolites concentration of adult males and females was 276.20±52.74 ng/g dry feces and 181.56±25.87 ng/g dry feces, respectively (Table 1). The concentrations of cortisol metabolites in adult males significantly higher compared to adult females (p<0.05). Moreover, the dominant male showed the highest levels of cortisol metabolites (Table 1).

These results indicated that the measurement of cortisol metabolites of Sambar deer can be performed non-invasively from feces using the 3α,11β-dihydroxy-etiocholanolone assay. This is because feces of most species of vertebrate contain metabolized forms of steroid hormones (i.e., progesterone, estrogen androgen and glucocorticoids/cortisol metabolites). These metabolized hormones are secreted into the gut via bile [12]. Although, these steroids in the gut are reabsorbed and transported to the liver (enterohepatic circulation) and the microbial flora metabolized these steroids, but the sterane skeletal structure is not degraded [22]. Thus, in the feces, the specific steroid metabolites can be then detected
by immunoassay technique. Therefore, it is possible to use feces to assess cortisol metabolites concentration for monitoring stress and animal welfare of Sambar deer in captivity.

Table 1. The cortisol metabolites concentrations (mean±SD) in feces of Sambar deer.

| ID of Sambar deer | Sex      | Status of Hierarchy | Cortisol metabolites concentration (ng/g feces) |
|-------------------|----------|---------------------|-----------------------------------------------|
| M1                | Adult male | Dominance          | 411.38±70.11                                  |
| M2                | Adult male | Subordinate        | 159.97±30.91                                  |
| M3                | Adult male | Subordinate        | 218.29±79.46                                  |
| F1                | Adult female | Subordinate    | 170.07±20.86                                  |
| F2                | Adult female | Subordinate    | 155.60±35.40                                  |
| F3                | Adult female | Subordinate    | 138.60±13.75                                  |
| F4                | Adult female | Subordinate    | 192.86±23.53                                  |

The higher concentration of cortisol metabolites in males than females seems related to the different social status of animals which dominant males showed the highest concentration of cortisol metabolites. According to Cavigelli and Caruso [23], males and females from various species form dominance hierarchies. Males often showed a male competition for access to food resources and mating activity. As result, males show frequent aggressive interactions than females. Different concentrations of cortisol metabolites in males and females can also be affected by visitor numbers and visitor activities. However, the effects may differ between the individual of Sambar deer.

3.2. The relationship between the number and arrival frequency of visitor with cortisol metabolites

The number of visitors showed a significant correlation with the cortisol metabolites concentrations ($r = 0.482, p < 0.05$) (Fig 1a). These results suggest that the increasing number of the visitor will increase the concentration of cortisol metabolites. In addition, the arrival frequency of visitors also showed a significant correlation with the cortisol metabolites concentrations ($r = 0.398, p < 0.05$) (Fig 1b). These results indicated that the more frequent visitors to Sambar deer’s enclosure, it will increase in the concentration of cortisol metabolites. Thus, both influence the increase of cortisol metabolites concentration. The increasing cortisol metabolites concentration to visitors’ presence is likely driven by fear due to the large visitor numbers and visitor activities (e.g., loud noises, sudden movement of visitors). Thus, Sambar deer may be threatened and as the result, they are retreating (hiding), movement inhibition and increasing the aggression [5]. These behaviors are fear indicators of animals when the presence of visitors [9].

The increase of cortisol metabolites concentration in feces of Sambar deer indicated that the Sambar deer is stressed. When the Sambar deer receives a stressor, it stimulates the hypothalamus-pituitary-adrenal (HPA) axis to produce cortisol. First, the hypothalamus secretes a neuropeptide hormone, corticotrophin-releasing-hormone (CRH) which stimulates the anterior pituitary to synthesize and secrete an adrenocorticotrophic hormone (ACTH). ACTH then stimulates the adrenal glands to produce cortisol [15]. These results indicated that cortisol metabolites can be used as an indicator of stress in this species. Although, there is evidence that the presence of visitors can have neutral (visitors do not affect animals) and positive effects in zoo’s animals [5], but many studies reported that the presence of visitors showed negative effects such as increased glucocorticoid (cortisol) in spider monkeys, *Ateles geoffroyii rufiventris* [24], Mexican wolves, *Canis lupus baileyi* [25] and blackbuck, *Antelope cervicapra L.* [26]. In addition, increased visitor numbers correlated with the aggression rates in gorillas [27], baboons, *Papio hamadryas* [7] and Indian gaur, *Bos gaurus gaurus* [8]. Therefore, efforts to educate human visitors are necessary. This can be performed by placing a warning sign for visitors (e.g., don't too close to animals, don't give food, don't noisy, etc). Besides that, zookeepers also have an important role to play in protecting animals from disturbance by visitors.
Figure 1. Correlation between the concentration of cortisol metabolites with (a) the number of visitors and (b) the arrival frequency of visitors.

Conclusion
The concentration of cortisol metabolites in Sambar deer can be measured non-invasively from feces using 3α,11β-dihydroxy-etiocholanolone assay. Moreover, cortisol metabolite concentration is associated with the number and arrival frequency of visitors.

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