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Analysis of $3\alpha,11\beta$ -dihydroxy-CM profile for the indicator of stress on male Javan rhinoceros

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Abstract. A study on Javan rhinoceros (*Rhinoceros sondaicus*) in Ujung Kulon National Park, Banten was done to monitor the levels of stress in their natural habitat. The study found that glucocorticoid hormone metabolite $3\alpha,11\beta$ -dihydroxy-CM from feces was suitable for indicating the levels of stress in Javan rhino. The assessment was done to study stress variations among the three rhinoceros that had different levels of feed intake, as well as to study variations of stress levels in dry and rainy seasons. The result from this study showed that there were fluctuations of $3\alpha,11\beta$ -dihydroxy-CM levels in two of the three rhinoceros. These fluctuations reflect the levels of stress associated with energy intake deficit (energy intake per body weight) and water deficit. This research shows that the deficit in energy intake per body weight, and water limitation are among the biggest sources of stress for rhino population. Stress originating from deficit of nutrients from food plan can be overcome by enriching the habitat of the Javan rhinoceros with high nutrient food plants such as stink vine (*Paederiascandens*), blackboard tree (*Alstoniascholaris*), and wild ginger (*Costusspeciosus*). Stress originating from water deficit can be mitigated by opening tracks and ensuring access to permanent year-round water sources.

Keywords: EIA, Javan rhinoceros, hormones, stress

1. Introduction

1.1. Individual Mechanisms and Responses on Stress

As in other mammal species, Javan rhinoceros' (*Rhinoceros sondaicus*) encounter conditions in their natural habitat that will directly or indirectly influence their physiological functions. Conditions that may cause an imbalance in the physiological functions are defined as a source of stress, or stressors [1]. Javan rhinoceros physiology responds to the stress through mechanisms involving neural signal transmission, endocrine system, as well as physiological adaptations. These processes are part of the stress response mechanism to maintain physiological balance (homeostasis) and are primarily controlled by the hypothalamus.

Mammals are able to respond to a variety of stressors from their external environment as well as from internal sources. Physical receptors on the rhinoceros' body surface can send signals to the pain

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center in the brain that relay stress signals to the hypothalamus [2]. Internally, chemical receptors can detect physiological changes such as blood composition (e.g., hypoglycemia, toxins) and nutritional composition, and send stress signals to the hypothalamus [3]. Upon reaching the hypothalamus, stress signals induce secretion of stress hormones such as adrenalin and cortisol. The stress response will also depend on the type of stress (acute or chronic). Stress response mechanisms may also be associated with changes of behavior, mediated by neuroendocrine processes involving signal transmission within the nervous system. This eventually leads to hormone secretion as well. Changes in behavior are part of an organism's "stress avoidance" strategy that allows animals to detect and avoid potential stressors [4]. Biochemical pathways in sending stress signals in mammals are summarised in Figure 1.

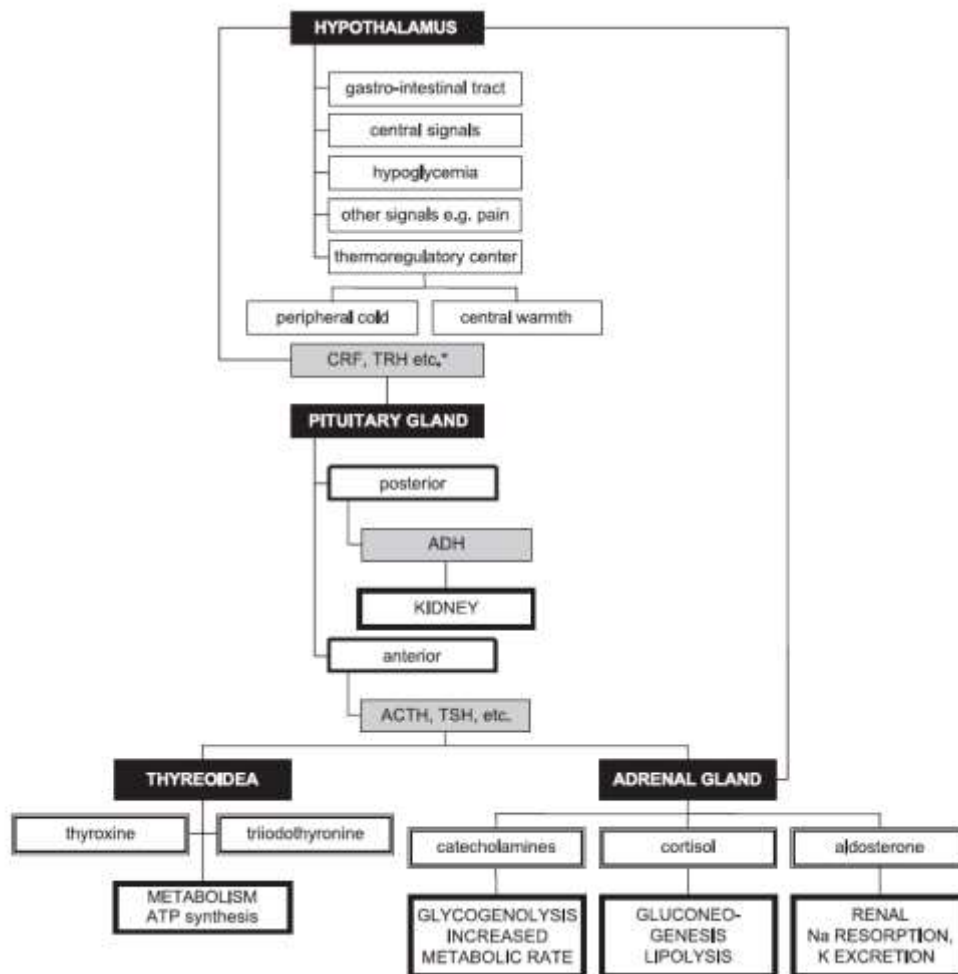


Figure 1. The biochemical process as a mammalian mechanism for responding to stress (source: Coenen 2005).

1.2. Definition of Stress

Acute, short-term stress is defined as a single pressure that is normally responded with a fight-or-flight response mediated by the sympathetic nervous system [4]. Chronic stress is defined as a long-term or repeated stress over time [5]. Both acute and chronic stresses stimulated hypothalamus activity that resulted in the increase of adrenal cortex activity, and increased glucocorticoid hormone secretion [6] in many animals, including rhinoceroses [7,8].

Chronic stress may occur due to the conditions faced by the Javan rhinoceros in their habitat in Ujung Kulon National Park. Food and water deficits may contribute to stress during the dry season. Climate change is anticipated to exacerbate stresses experienced by the Javan rhinoceros during the dry season by decreasing the availability of water sources [9].

1.3. Stress Hormone and Its Metabolites

Concentration of stress hormones from glucocorticoid groups have been widely used as indicators of stress levels in animals such as dogs [10], carnivores (Himalayan black bear, sloth bear, domestic cat, cheetah, clouded leopard, black-footed ferret, slender-tailed meerkat, and red wolf) [11], water buffalo [12], rodents [13], and cows [14]. Cortisol is a stress hormone produced by the adrenal cortex of the adrenal gland and is excreted through feces or urine after its use in gluconeogenesis and lipolysis (See Figure 1) and therefore stress levels can also be measured from the concentrations of the hormone metabolites excreted through an animal's feces and/or urine. Glucocorticoid hormone metabolites such as androstenediol increase in parallel with an increase of stress experienced by the animal due to increased adrenal cortex activity [6]. However, there is a possibility that the excreted metabolite has experienced inactivation where metabolic conversion, conjugation, and reduction occur. Therefore, methods for detecting the metabolite concentration need to ensure that, despite the inactivation, the metabolite still has a positive correlation with the actual blood concentration of cortisol or corticosterone [15]. A cortisol metabolite commonly used as an indicator of stress in animals is $3\alpha,11\beta$ -dihydroxy-CM [16].

2. Research Objectives

This research was designed to test the hypothesis that stress hormone metabolite from a fecal sample of Javan rhinoceros can be used as an indicator for stress level, based on the glucocorticoid profiles. Considering the potential use of glucocorticoid from fecal sample, and the needs to monitor the stress level among individual Javan rhinoceros, this research was designed to achieve the following objectives:

- Identifying potential indicators for stress hormone glucocorticoid from feces of Javan rhinoceros;
- Examining the characteristics of glucocorticoid profile extracted from a fecal sample of Javan rhinoceros; and
- Analyzing the correlation of stressors (i.e., water deficit) with glucocorticoid profile extracted from feces of Javan rhinoceros.

3. Methods

3.1. Sample Javan Rhinoceros

Two adult males and one young male Javan rhinoceros were selected for this research, as they had been involved in the nutrition study [17].

3.2. Defining Stressors in the Habitat of Javan Rhinoceros

Sources of stress in the natural habitat of Javan rhinoceros in Ujung Kulon National Park were primarily originated from two factors, consisting of food intake (nutrition), and water availability. Food intake measured as protein, fat, and energy did not show significant variation among the sample rhinos [17], so water availability was selected as a stressor that would provide a more obvious stress response. Water availability was represented in the dry and wet seasons, which were differentiated by low and high rain occurrences, respectively. The number of days with rain (rain occurrences) were recorded every month throughout the research period from October 2009 to April 2010.

3.3. Defining Criteria of Fecal Samples

Hormone measurement and assessment needed to rely on the glucocorticoid from the fecal sample. This assessment was done using hormone assessment from a fecal sample [18], as performed on wild dog

species. The same concept was applicable for Javan rhinoceros with fecal samples that were in relatively fresh condition (ideally within 24 hours, but no more than 30 hours after excretion). Fresh fecal samples should be in ball shapes (bolus) and had a greenish-brown color with moisture (mucous) on the surface. Additionally, the fresh fecal samples were still surrounded by flies.

3.4. Collection of Fecal Samples

To ensure fresh sample collection, individual Javan rhinoceros were followed at a safe distance throughout the observation period (October to January). Any feces found during the collection period were immediately collected. Five grams of feces sample were collected using a wooden spoon and was placed in a 50-ml plastic vial. The sample was fixed by immersing it in 25 ml of 90% ethanol (technical grade). The samples were sent to Rehabilitation and Reproduction Unit Laboratory at Bogor Agriculture University, where they are stored in the freezer at a temperature of -20 °C.

3.5. Selection of Hormone Metabolites from Fecal Samples

Hormone assay was done using enzyme immunoassay (EIA) kits for cortisol (DRG Germany), corticosterone (CUSABIO, China), and $3\alpha, 11\beta$ -dihydroxyetiocholanolone ($3\alpha, 11\beta$ -dihydroxy-CM) developed by German Primate Centre di Göttingen, Germany. The hormone assay was done at Rehabilitation and Reproduction Unit Laboratory, Bogor Agriculture University. The parallelism test was conducted as a standard procedure for determining the consistency of antigen-antibody bonds provided within each EIA kit [19]. Suitability for such assay using fecal samples of Javan rhinoceros was also determined from this test. Parallel curves for glucocorticoid metabolite should be produced when tested with different (cascading) concentrations. In addition to validating the suitability of assay kit, variations within the same kit (intra-assay variations), and also across different kits (inter-assay variations) [20]. As in EIA of African white rhinoceros (*Ceratotherium simum*), the optimal dilution factor with 50% antigen-antibody binding was determined for each kit [21]. The optimal dilution factor would provide the most accurate measurement of glucocorticoid from fecal sample.

Additionally, cross-reactivity between antibodies with different antigens would disrupt the accuracy of the assay, as it would provide inconsistent binding resulting in an inconsistent measurement of metabolite [22]. For instance, 5-beta-androstenediol was a glucocorticoid metabolite with low cross-reactivity with other hormones. 5-beta-androstenediol had 0.5% cross-reactivity with testosterone, and 1.49% cross-reacts with androstenedione used in testosterone assay [23]. EIA kit for $3\alpha, 11\beta$ -dihydroxy-CM was never been used with fecal samples of Javan rhinoceros. Therefore, potential issues due to cross-reactivity with androgen were anticipated, as cortisol and other 19-carbon androgens could also be detected by this kit.

3.6. Preparation of Fecal Sample for Hormone Assay [24]

Metabolite extraction was done by thawing the frozen sample until the 90% ethanol (technical grade) was back to a liquid state, and was completely evaporated. The dried fecal sample was mixed with 2.5 ml of 90% ethanol and 2.5 ml of distilled water. This solution was thoroughly mixed in the plastic vial. The solution was centrifuged for five minutes to separate the solvent with the solid particle. The solid particle was an extract for subsequent use in hormone assay (EIA) to measure cortisol, corticosterone, and $3\alpha, 11\beta$ -dihydroxy-CM.

3.7. Cortisol Assay

Cortisol assay was done according to the procedures stated in the assay kit produced by DRG, Germany. The kit provided 96 microtiter wells coated with monoclonal anti-cortisol antibody. Cortisol assay using this kit provided detection range of 0-800 ng/mL, and concentration for standard curves at 800 ng/ml, 400 ng/ml, 200 ng/ml, 50 ng/ml, 20 ng/ml, and 0 ng/ml. Cross reactivities with Cortisol (100%), Corticosterone (45%), Progesterone (95%), Deoxy cortisol and Dexamethasone (2% each), estrone, estriol, and testosterone (below 0.01% each) were identified; assay sensitivity was 2.5 ng/ml (6.9

nmol/l); intra-assay precision was 3.2%-8.1% (based on 20 repetitions); and inter-assay precision was 6.6%-7.7% (based on 20 repetitions).

3.8. Corticosterone Assay

Kit for corticosterone was available from CUSABIO, China. A detailed assay procedure was described in this kit. Microtiter plate with 96 wells coated with monoclonal anti-corticosterone antibody was included in the kit. Corticosterone assay had a range of 0.6-160 ng/ml, and the concentration for standard curves of 160 ng/ml, 40 ng/ml, 10 ng/ml, 2.5 ng/ml, and 0.63 ng/ml. There was no known cross-reactivity with other hormone molecules. Assay sensitivity was below 0.4 ng/ml; intra-assay precision is below 10% (20 repetitions); and inter-assay precision was below 8% (based on 35 repetitions).

3.9. $3\alpha,11\beta$ -dihydroxy-CM Assay

$3\alpha,11\beta$ -dihydroxy-CM assay was done according to the procedures stated in the assay kit produced by the German Primate Centre in Gottingen. The kit provided 96 microtiter wells coated with monoclonal anti- $3\alpha,11\beta$ -dihydroxy-CM antibody. Assay for $3\alpha,11\beta$ -dihydroxy-CM assay had a concentration range of 0.6 ng/ml to 78 ng/ml. Standard curve concentrations were defined at 78 ng/ml, 39 ng/ml, 19.5 ng/ml, 9.6 ng/ml, 4.8 ng/ml, 2.4 ng/ml, 1.2 ng/ml, and 0.6 ng/ml. There was a potential cross-reactivity with androgen (testosterone). Sensitivity of this assay was 2.5 ng/ml (6.9 nmol/l) with intra-assay precision of 4.4%-5.4% (18 repetitions); and inter-assay precision was determined at 6.8%-11.2% (based on 6 repetitions).

3.10. Data Analysis

Gen5 software (developed by Biotek, Germany) was used to construct standard curves based on all the three EIA assays. Comparisons of standard curves according to the specifications of each kit manufacturers were used for parallelism test to determine consistencies of hormone assay on fecal samples of Javan rhinoceros. Consistency was shown by assay(s) with parallel (non-crossing), and assay(s) were selected based on this consistency for measuring stress hormone.

Variables used for analysis were rhinoceros consuming food plants with high and low nutrition (energy) in the same season (rhinoceros 12 and 13 have a high-energy intake, while rhinoceros 18 has low energy intake); and all three rhinoceros in dry and wet seasons. Metabolites' concentration from fecal samples (in ng/mL) was used to indicate the differences in stress levels across different variables. Based on daily rain occurrences, samples from October were considered to represent the dry season, while samples from January were considered to represent the wet season. Due to the low numbers of samples, descriptive statistics were used to analyze these differences.

4. Result and Discussion

4.1. Result of Parallelism Test

Results of the parallelism test exclude cortisol and corticosterone from potential kits for measuring the metabolites from fecal samples of Javan rhinoceros. Standard curves of cortisol and corticosterone kits were not parallel (some curves are crossing with those at different concentrations). In contrast, assay using glucocorticoid metabolism $3\alpha,11\beta$ -dihydroxy-CM show parallel standard and sample curves. Parallelism test using $3\alpha,11\beta$ -dihydroxy-CM suggests 50% of binding at 3.04 pg concentration and 1:80 dilution. Subsequent validation also shows intra-assay variations of 4.4% to 5.4% from 18 repetitions. Inter-assay variations show 6% to 11.2% based on 6 repetitions. Variations of both intra and inter assays show values lower than 15%, so it can be concluded that $3\alpha,11\beta$ -dihydroxy-CM is suitable for measuring the glucocorticoid profiles based on fecal samples of Javan rhinoceros.

4.2. Dilution factor for Quantification of Glucocorticoid Metabolites ($3\alpha,11\beta$ -dihydroxy-CM)

Standard and sample curves from the parallelism test in Figure 2 are used to determine the optimal dilution factor for measuring metabolites from fecal samples of Javan rhinoceros. The optimal dilution factor is obtained at 50% antigen-antibody binding. In the case of $3\alpha,11\beta$ -dihydroxy-CM, 50% binding is achieved at a 1:80 dilution factor.

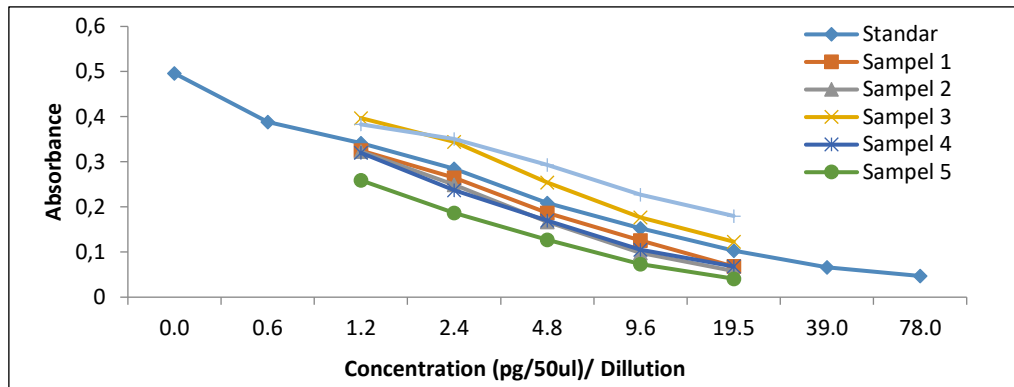


Figure 2. The standard curve replicates for $3\alpha,11\beta$ -dihydroxy-CMkit with different dilutions on fecal samples of Javan rhinoceros.

4.3. Profile of $3\alpha,11\beta$ -dihydroxy-CM on individual rhinoceros

Fluctuations of glucocorticoid concentrations of all rhinoceros in different months are described in Figure 3. Glucocorticoid concentrations show high values (corresponding with high-stress hormone concentration in blood) in October and November, indicating that the individuals experienced higher stress conditions during these months. In contrast, the value is low in January and February. Additionally, to assess the glucocorticoid profile in different rhinoceros individuals, box plot analysis (Figure 4) is done to verify if there is a significant difference. Box plot analysis suggests no significant differences among individual rhinoceros, but individual number 18 showed high variations of stress levels throughout the period.

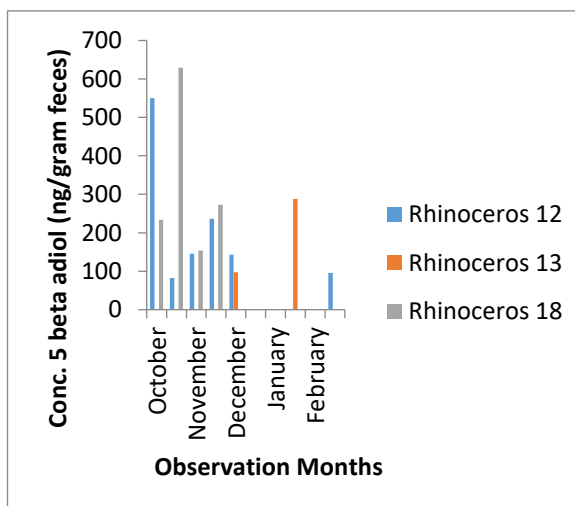


Figure 3. Fluctuations of $3\alpha,11\beta$ -dihydroxy-CM levels in all rhinoceros in different months (seasons).

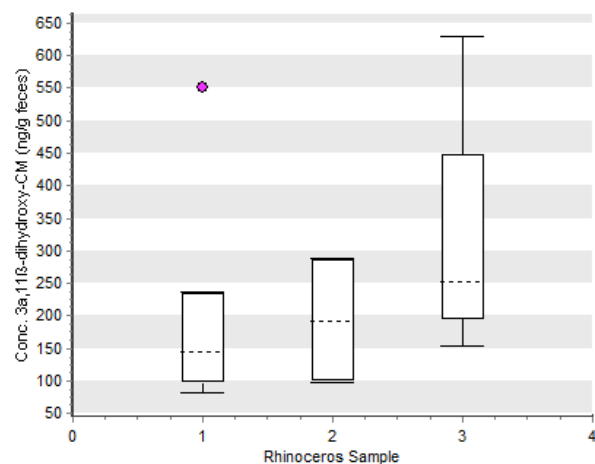


Figure 4. Range of glucocorticoid metabolite ($3\alpha,11\beta$ -dihydroxy-CM) concentration in three different individual rhinoceros.

4.4. Stress Factors

October and November are marked with notably higher glucocorticoid metabolites compared to those in January and February. This may be associated with the differences in water availability during these observation months. Field observation confirms that dry season occurs in October when the average daily rain occurrence average is 0.2 per day (average of every five days rain). In January, the average daily rain occurrence is 1.61 per day (average of more than one rain occurrence per day). Fewer rain occurrences are likely to result in lower overall rainfall, which results in a decrease in water availability [25]. By comparing the glucocorticoid data with rain occurrences, it can be inferred that the stress level is relatively higher during the dry season in October than in the wet season in January.

Rhinoceros number 18 (young/sub-adult rhinoceros) shows the higher median of $3\alpha, 11\beta$ -dihydroxy-CM compared to the other two adult rhinoceros. This suggests that rhinoceros number 18 experience a higher level of stressors throughout the research period. Higher levels of stressors may occur, as the young individual is establishing its own home range[26]. A high level of metabolites may also be associated with a high testosterone level. Increased testosterone in mammalian species is often associated with spermatogenesis process observed at the beginning of the wet season[21]. However, $3\alpha, 11\beta$ -dihydroxy-CM has a high probability of cross-reactivity with androgen (i.e., testosterone). Testosterone also fluctuates with seasonal changes[27].

Additional stress factors may include nutritional aspects. Rhinoceros number 18 had low energy intake per body weight [17] indicates high $3\alpha, 11\beta$ -dihydroxy-CM in feces. Such low intake may cause an energy deficit that triggers the glucocorticoid hormone needed for gluconeogenesis and lipolysis. Correlation between energy intake and level of $3\alpha, 11\beta$ -dihydroxy-CM in feces from this research is shown in Figure 5, which suggests that there is a negative correlation between energy intake and glucocorticoid level, where high glucocorticoid level correlates with low energy intake.

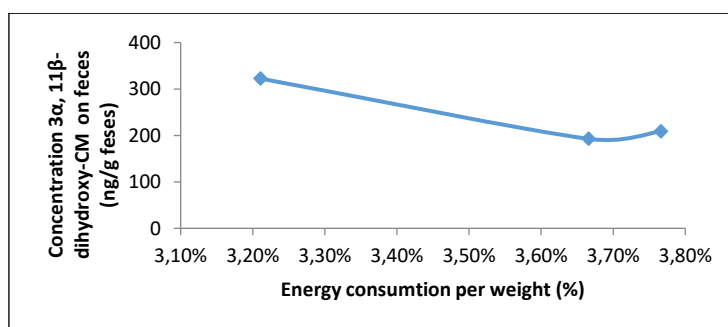


Figure 5. Correlation between energy consumption and concentration of glucocorticoid metabolite in feces.

5. Summary and Suggestion

5.1. Summary

1. Analysis of Javan rhinoceros fecal samples using EIA for $3\alpha, 11\beta$ -dihydroxy-CM shows parallel results between standard and sample curves under cascading dilution. This suggests the $3\alpha, 11\beta$ -dihydroxy-CM assay is appropriate for detecting glucocorticoid metabolites in Javan rhinoceros fecal samples;
2. Glucocorticoid levels tended to vary throughout the research period. This suggests a seasonal variation of stress due to water availability in dry and wet seasons. Stress tends to be higher in the dry season compared to the wet season, likely due to decreased water availability;
3. A young (sub-adult) male rhinoceros shows higher variations of stress that can be caused by stressors from habitat conditions (food and water availability), as well as intra-specific competition with adult rhinoceros when the young male is expanding its home range;

4. Higher levels of glucocorticoids were correlated with a low level of energy intake from food; and
5. The results concluded that environmental factors such as water availability and food quality (energy) are crucial stressors for the Javan rhinoceros.

5.2. Suggestions

1. Further validation to ensure the suitability of 3 α ,11 β -dihydroxy-CM for measuring glucocorticoid metabolite in the fecal sample of Javan rhinoceros. Validation processes needed include assessing the probability of cross-reactivity with androgen. Samples from female Javan rhinos are needed for this validation, as female rhinoceros do not have androgen that will interfere with the glucocorticoid readings;
2. Develop longitudinal studies to monitor fluctuations of physiological stress in Javan rhinoceros based on fecal samples (non-invasive method) using the most appropriate hormone assay;
3. Study the impact of stress on the reproduction of Javan rhinoceros in their natural habitat;
4. Improving food quality by enhancing the feeding ground with high-energy food plants such as stink vine (*Paederia scandens*), blackboard tree (*Alstonia scholaris*), and wild ginger (*Costus speciosus*); and
5. Mitigating stress due to water deficit by opening tracks and ensuring access to food plants with high water content, and access to permanent year-round water sources.

6. References

- [1] Morgan K N and Tromborg C T 2007 Sources of stress in captivity *Appl. Anim. Behav. Sci.*
- [2] Baron R 2006 Mechanisms of disease: Neuropathic pain - A clinical perspective *Nat. Clin. Pract. Neurol.*
- [3] Coenen M 2005 Exercise and stress: Impact on adaptive processes involving water and electrolytes *Livestock Production Science*
- [4] Dickens M J, Delehanty D J and Michael Romero L 2010 Stress: An inevitable component of animal translocation *Biol. Conserv.*
- [5] Wingfield J C and Romero L M 2011 Adrenocortical Responses to Stress and Their Modulation in Free-Living Vertebrates *Comprehensive Physiology*
- [6] Barja I, Silván G, Rosellini S, Piñeiro A, González-Gil A, Camacho L and Illera J C 2007 Stress physiological responses to tourist pressure in a wild population of European pine marten *J. Steroid Biochem. Mol. Biol.*
- [7] Turner J W, Tolson P and Hamad N 2002 Remote assessment of stress in white rhinoceros (*Ceratotherium simum*) and black rhinoceros (*Diceros bicornis*) by measurement of adrenal steroids in feces. *J. Zoo Wildl. Med.*
- [8] Menargues A, Urios V and Mauri M 2008 Welfare assessment of captive Asian elephants (*Elephas maximus*) and Indian rhinoceros (*Rhinoceros unicornis*) using salivary cortisol measurement *Anim. Welf.*
- [9] Permadi Y 2008 *Kajian dampak perubahan iklim terhadap kerentanan badak Jawa* (Jakarta)
- [10] Schoeman J P, Goddard A and Herrtage M E 2007 Serum cortisol and thyroxine concentrations as predictors of death in critically ill puppies with parvoviral diarrhea *J. Am. Vet. Med. Assoc.*
- [11] Young K M, Walker S L, Lanthier C, Waddell W T, Monfort S L and Brown J L 2004 Noninvasive monitoring of adrenocortical activity in carnivores by fecal glucocorticoid analyses *Gen. Comp. Endocrinol.*
- [12] Garg S and Chander S 1997 Plasma Cortisol and thyroid hormone Concentrations in Buffaloes with uterine Torsions *Buffalo Bulletin* **16** 75–6
- [13] Soto-Gamboa M, Gonzalez S, Hayes L D and Ebensperger L A 2009 Validation of a radioimmunoassay for measuring fecal cortisol metabolites in the hystricomorph rodent, octodon degus *J. Exp. Zool. Part A Ecol. Genet. Physiol.*

- [14] Morrow C J, Kolver E S, Verkerk G A and Matthews L R 2002 Fecal glucocorticoid metabolites as a measure of adrenal activity in dairy cattle *Gen. Comp. Endocrinol.*
- [15] Wasser S K, Hunt K E, Brown J L, Cooper K, Crockett C M, Bechert U, Millspaugh J J, Larson S and Monfort S L 2000 A generalized fecal glucocorticoid assay for use in a diverse array of nondomestic mammalian and avian species *Gen. Comp. Endocrinol.*
- [16] Ghalib, Supriatna I, Agil M and Engelhardt A 2011 *Non-invasive hormone monitoring: fecal androgen and glucocorticoid in male crested macaques (Macaca nigra) in relation to seasonal and social factors* (Bogor Agriculture University)
- [17] Hariyadi A R S, Sajuthi D, Astuti D A, Alikodra H S and Maheshwari H 2016 Analysis of nutrition quality and food digestibility in male Javan rhinoceros (*Rhinoceros sondaicus*) in Ujung Kulon National Park *Pachyderm* **57** 86–96
- [18] Santymire R M and Armstrong D M 2010 Development of a field-friendly technique for fecal steroid extraction and storage using the African wild dog (*Lycaon pictus*) *Zoo Biol.*
- [19] François-Gérard C, Gérard P and Rentier B 1988 Elucidation of non-parallel EIA curves *J. Immunol. Methods*
- [20] Schoenecker K A, Lyda R O and Kirkpatrick J 2004 Comparison of Three Fecal Steroid Metabolites for Pregnancy Detection Used with Single Sampling in Bighorn Sheep (*Ovis canadensis*) *J. Wildl. Dis.*
- [21] Kretzschmar P, Gansloßer U and Dehnhard M 2004 Relationship between androgens, environmental factors and reproductive behavior in male white rhinoceros (*Ceratotherium simum simum*) *Horm. Behav.*
- [22] Agil M, Setiadi D, Supriatna I and Purwantara B 2008 Non-Invasive Endocrine Monitoring of Reproduction and Stres in the wild animal: Analyzing hormone metabolites in Urine and Faeces using Enzymeimmunoassay *Proceeding of AZWMC*
- [23] Brown G A, Martini E R, Roberts B S, Vukovichand M D and King D S 2002 Acute hormonal response to sublingual androstenediol intake in young men *J. Appl. Physiol.*
- [24] Ziegler T E and Wittwer D J 2005 Fecal steroid research in the field and laboratory: Improved methods for storage, transport, processing, and analysis *American Journal of Primatology*
- [25] Smit I P J and Grant C C 2009 Managing surface-water in a large semi-arid savanna park: Effects on grazer distribution patterns *J. Nat. Conserv.*
- [26] Hearne J W and Swart J 1991 Optimal translocation strategies for saving the black rhino *Ecol. Modell.*
- [27] Paplinska J Z, Moyle R L C, Wreford N G, Temple-Smith P D M and Renfree M B 2007 Reproduction in male swamp wallabies (*Wallabia bicolor*): Puberty and the effects of season *J. Anat.*