Indonesian Journal of Veterinary Sciences
Vol. 4. No. 1. March 2023, Page. 39-44
DOI:10.22146/ijvs.v4i1.90905, ISSN 2722-421X (Online)
Available online di https://journal.ugm.ac.id/ijvs

The Effect of the Number of Visitors on Testosterone Levels in the Feces of Horses (*equus caballus*) at Mini Zoo Jogja Exotarium

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Received: November 21, 2022, Accepted: January 13, 2023, Published: March 1, 2023

Abstract

Agro-tourism is a type of tourism that is currently in demand by tourists. One of the popular agro-tourism concepts is the one that carries the concept of a combination of animal farming and agriculture. This concept can be found at the Mini Zoo Jogja Exotarium. Mini Zoo Jogja Exotarium offers facilities for visitors to interact with various kinds of animals, including horses. This study aimed to examine the effect of the number of visitors at Mini Zoo Jogja Exotarium on testosterone levels in horse feces samples.The fecal samples that were being used were non-invasively collected for 12 days. Horse fecal samples were extracted using 80% methanol and then analyzed quantitatively using the Enzyme-Linked Immunosorbent Assay (ELISA) method. The results showed that the lowest horse testosterone level was 52.52 ng/gr dry feces when the number of visitors reached 199 people, while the highest testosterone level was 168.43 ng/gr dry feces when there were 26 visitors. The statistical analysis results show that both variables have -0.298 as a Correlation Coefficient. Based on the two analysis results, it can be concluded that the number of visitors has a weak and negative correlation with the metabolism testosterone horse.

Keywords: ELISA; feces; Horse; non-invasive; testosterone

Introduction

Agrotourism is a concept of tourism objects that are now popular with tourists. According to Joshi *and* Bhujbal (2012), agrotourism is a form of tourism in rural areas and aims to provide visitors with an experience of closeness to nature in agriculture-based activities. Putritamara et al. (2020) stated that agrotourism was established to provide educational, entertainment, cultural, and business benefits related to agriculture and animal husbandry. Tourists can see the animals' way of life and various methods of caring for them. The large number of tourists visiting can affect the normal physiological condition of animals. This is because direct interaction between visitors and animals can interfere with animal comfort.

Reproductive function is one of the many bodily functions that can be affected by environmental conditions. The hormone testosterone can indicate the reproductive activity of male animals (Astuti *et al.,* 2006). It can fluctuate depending on the mechanism of LH hormones from the pituitary and GnRH from the hypothalamus (Muller and Lipson, 2003). When the animal's condition is disturbed, the pituitary will be triggered to secrete Adrenocorticotropic Hormone (ACTH) and impact increasing cortisol levels from the adrenal cortex. An increase in cortisol will cause a decrease in testosterone levels produced by Leydig cells. Therefore, cortisol is also a physical factor that lowers testosterone production (Hidayatullah *et al.,* 2018).

Horse is Animals that have always been close and often used by humans; even now, horses are used as attractions in tourist centers. Therefore, this study aims to determine the effect of the tourist environment on the physiological status of horse animals and know the level of welfare of animals kept in a conservation environment. According to Sherwen and Hemsworth (2019), if animals perceive humans as enemies (predators or competition), it can cause a fear response and impact poor levels of animal welfare. In addition, interference from the environment also causes an increase in the secretion of Adrenocorticotropic Hormone (ACTH) so that the adrenal glands produce more glucocorticoids. The resulting glucocorticoids will form bonds with Leydig cells and impact decreasing testosterone levels (Faldikova, et.al*.,* 2001).

Materials and Methods

Time and place

The research conducted includes three stages, namely the stool sampling stage, the preparation stage, and the analysis stage. The stool samples of stallions used were taken from Mini Zoo Jogja Exotarium at Jalan Magelang km. 8, Sendangadi Village, Mlati District, Sleman Regency, Yogyakarta Province. The sampling lasted twelve days, from April 19 to 30, 2021. Using the ELISA method, the stool samples were extracted using 80% methanol for further analysis.

Sample

The sample used in the study was a stool sample from a stallion (*Equus caballus*), a 10-year-old Sumbanese breed kept in a cage. Data on the number of visitors is obtained from the record of daily ticket sales.

Materials

Tools and Materials used during the study include plastic clips, markers, latex gloves (gloves), plastic tube, freeze dryer (Labfreeze® FD-10MR)*,* pounding tools, measuring spoons, scales, 15 ml conicle tubes, pipettes, beakers, vortexes, micropipettes and tips, Eppendorf tubes, Calbiotech ELISA kits® Testosterone, ELISA washing machine (ELISA-Washer) (Zenix Diagnostic®, Model ZN-390), ELISA-Reader (Zenix Diagnostic®, Model ZN-320)*,* and 80% methanol.

Methods

Ethical clearance

Although the research uses non-invasive methods, Which means there is no animal treatment, submissions are still made ethical clearance to the ethics commission of Integrated Laboratory for Research and Testing Universitas Gadjah Mada. This aims to ensure that the procedures carried out are to the requirements and ethics in using experimental animals. Certificate ethical clearance LPPT UGM published this research with number 00043/04/ LPPT/X/2021.

Visitor Data

The number of visitor data was obtained from recording ticket sales during sampling from 18 to 30 April 2021.

Sample collection

Samples are collected once per day; sampling is carried out between 09.00-13.00 on April 19-30, 2021. The stool is put into plastic clips to be stored in a Freezer. Stool samples stored in the freezer inserted into the next conical tube, a freeze-dryer process is carried out for 3 x 24 consecutive hours. Astuti et al. (2006) stated that the freeze-drying process is conducted for the lyophilization of samples, ensuring that the sample remains dry without damaging its contents. The feces are ground until smooth and taken as much as 0.5 g, then 80% methanol, as much as 5 ml, a mixture of fecal powder and methanol, then homogenized using vortex for 10 minutes. Using the EIA method, supernatants in the upper layers are taken to analyze testosterone levels.

Hormone Detection

Testosterone testing according to Calbiotech ELISA Kit manual®. The procedure begins by inserting a standard solution and a sample of 25 µl each, then adding 50 µl of enzyme conjugate and 50 µl of Biotin reagent to each contribution. The next stage is the incubation microplate. For

60 minutes, after the incubation process is complete, all the liquid is removed from the well, and then the microplate is washed three times using a 300 µl Buffer washer on ELISA-Washer. After washing and drying the microplate on tissue paper, 100 µl of reagent Tetramethylbenzidine (TMB) was added to each contribution. The microplate was covered and incubated at room temperature for 30 minutes, and then 50 µl was added stop solution. The intensity of the resulting color is then read using an ELISA-Reader with a wavelength of 450 nm.

Results and Discussion

Sampling for testosterone level tests can be done non-invasively or invasively. Astuti et al. (2006) stated that non-invasive methods are more beneficial when compared to invasive methods. According to Kumar et al. (2013), non-invasive methods do not make animals experience stress, prioritize animal welfare, and are easy to do in the field. The results obtained from non-invasive samples are also not influenced by handling procedures such as capture, restraint, and anesthesia, so the circulating testosterone steroid hormone does not change its concentration (Pribbenow *et al.,* 2016). Sampling is done at that hour because testosterone is a hormone that has a diurnal rhythm so that it is secreted higher in the morning (Ganiswarna, 1995) and decreases during the day to night (Winter, 1999).

Testosterone levels are measured using horse fecal samples collected by non-invasive for twelve days. Samples are processed and analyzed using Enzyme-Linked Immunosorbent Assay (ELISA) methods. According to Gholib et al. (2016), commercial ELISA kits are generally not designed for animals, so validation tests need to be carried out first if they are to be used for animal hormone analysis. The validation test aims to measure the kit's accuracy and precision so that it can be decided whether the kit is acceptable for measuring the hormone testosterone in the species to be tested. According to Gholib et al. (2020), validation tests can be conducted analytically, physiologically, and biologically. In the study, analytical validation was carried out through parallelism tests. This test is carried out by diluting the sample in stages with a ratio of 1:10 to 1:800 using sterile aquadest. According to Gholib et al. (2016), diluted samples were tested with testosterone standards (standard serial dilution of testosterone 0.2-16 ng/ml). However, in this study, a 0.2-18 ng/ml dilution was carried out (Table 1 and Figure 1).

Table 1. Results of serial dilution of testosterone standard.

Standard	Testosterone levels (ng/ml dry stool)	OD 1	OD 2	Average OD
A	0	1,481	1,432	1,4565
B	0,2	1,281	1,167	1,224
C	0,5	1,0006	0,993	0,9968
D	2	0,713	0,545	0,629
E	6	0,338	0,404	0,371
F	18	0,153	0,139	0,146

Figure 1. Graph of the raw curve resulting from the serial dilution of standard testosterone and the parallelism test curve

According to Fernanda et al. (2019), verification of the linearity of the standard curve is needed before determining testosterone levels using ELISA so that it can be known whether or not there is an effect of changes in standard levels on Optical Density. Table 1 shows mean OD progressively decreases at higher testosterone concentrations (inversely proportional). The parallelism test curve graph also looks to have a slope similar to the standard curve.

Based Table 2., the lowest horse testosterone level was 52.52 ng/gr dry feces when the number of visitors reached 199 people, while the highest testosterone level was 168.43 ng/gr dry feces when there were 26 visitors. Analysis statistic shows a value *Correlation Coefficient* Of -0.298; this can be interpreted if one variable increases, then the other variable will decrease. The number of daily visitors can be categorized into factors that affect animals' physiological and emotional states. According to Palme *et al.* (2005), if animals are faced with physical or emotional stress, they will rely on their biological systems to respond, one of which is reflected in their neuroendocrine response. When an animal is disturbed by its environment, the hypothalamus will release *Corticotrophic Releasing Hormone* (CRH), which causes the

Table 2. OD values, testosterone levels of the sample, and the number of visitors per day for twelve days.

Day and Date	OD	Testosterone levels (ng/gr dry fecal)	Number of Visitors (people)
Monday, 19 April 2021	0,168	149,41	50
Tuesday, 20 April 2021	0,151	160,28	24
Wednesday, 21 April 2021	0,193	134,74	27
Thursday, 22 April 2021	0,183	140,43	36
Friday, 23 April 2021	0,255	104,29	43
Saturday, 24 April 2021	0,255	104,29	90
Sunday, 25 April 2021	0,421	52,52	199
Monday, 26 April 2021	0,139	168,43	42
Tuesday, 27 April 2021	0,188	137,56	58
Wednesday, 28 April 2021	0,309	83,43	21
Thursday, 29 April 2021	0,147	162,95	29
Friday, 30 April 2021	0,107	192,24	34

anterior pituitary to release *Adrenocorticotropic Hormone* (ACTH). The presence of ACTH in circulation impacts increasing cortisol hormone levels (Hunt, et.al., 2019). Soaring cortisol levels will inhibit GnRH release and production *of Luteinizing Hormone* (LH) so that testosterone reproductive hormone levels decrease (Hidayatullah *et al.,* 2018).

The results of the Correlation Coefficient of -0.298 show that the number of daily visitors at Mini Zoo Jogja Exotarium and testosterone levels of horse feces has a weak relationship. This follows Sherwen and Hemsworth's (2019) statement that animals that have been domesticated can adapt to humans and conserve maintenance environments due to genetic changes from generation to generation. The absence of the influence of the number of visitors on testosterone levels can indicate a neutral relationship between the two. Animals have become accustomed to the presence of visitors, causing the perception that visitors are part of life or part that does not threaten the environment. The animal's familiarity with human presence can also be caused by the presence of both keepers all the time, which has an impact on the positive response of the horse, following Martin and Melfi's (2016) statement that animal proximity to humans plays a crucial role in animal behavioral responses to humans and has the potential to affect the quality-quantity of interactions and relationships produced as well as overall animal welfare. This is because keepers interact a lot with animals daily when feeding, cleaning cages, and training animals, so this interaction will develop into a relationship (Sherwen & Hemsworth, 2019).

According to Digard (1999), horses have been domesticated to be used as transportation animals since ancient times. According to Gill et al. (2019), horses are gentle, friendly, and social. In addition, in a controlled environment, his behavior is easy to predict. Based on research conducted by Seaman et al. (2002), there was no influence of human perspective on the reaction shown by horses. Horses show the same response in approaching humans with or without visual contact. Hama et al. (1996) state that visitors who show neutral or positive feelings when petting horses do not impact the physiological status of horses. This is because the response horses show is directly proportional to the attitude of humans who make contact (Chamove *et al.,* 2002). This may explain the insignificant relationship between the two variables: the number of visitors and testosterone levels. As domesticated animals, horses have gone through a long process to get used to the human state around them. In addition, the horses studied have been kept for more than ten years, so they have had various positive experiences with keepers and humans. Several studies on the influence of tourists have revealed that the reaction of horses when interacting with humans is primarily a blend of temperament human skills in handling horses, as well as experiences gained with humans (Hausberger et al. (2008); visitor pressure did not negatively affect the welfare

of European bison as there was no increase in stress hormones in the collected fecal samples (Klich *et al.,* 2021); ruminants kept in zoos show little reaction to visitors, but in some species the reaction shown is sex-dependent (Thompson, 1989); in black-tailed prairie dogs (*Cynomys ludvicianus)* showed positive behavior when there were more visitors at The Saint Louis zoo, Missouri, USA (Eltorai & Sussman, 2010); and chimpanzees (*Pan troglodytes)* were observed to show positive behavioral changes in the presence of visitors (Wood, 1998).

This study used stallions that were stabled most of the time but still exercised and indulged in the field for a specific time every day. Horses are not used as riding horses. It interacts less with visitors than horses used for horseback riding facilities; visitors can still interact with horses, but it is constrained to only being done outside the stable. According to Davey (2007), other factors such as density, interaction activity, and visitor position can affect animal behavior and physiological changes. The location of the enclosure where animals are exhibited also affects because visitors will be more interested in seeing animals near the entrance (Mitchell et al., 1990). In addition, the number of visitors calculated from the number of tickets sold cannot represent the number of visitors who interact with animals (Klich et al., 2021).

Conclusion

Based on the results of the study, it can be concluded that there is no correlation between the number of visitors and testosterone levels in the feces of stallions..

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