Positive reinforcement conditioning as Sumatran tiger's (*Panthera tigris sumatrae*) social enrichment at Tambling Wildlife Nature Conservation Rescue Centre, Lampung, Indonesia

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Abstract. *Kiranaputri G, Sjahfirdi L, Tumbelaka LITA, Yana A, Priyanto SK, Anggarsari LY, Marizal. 2021. Positive reinforcement conditioning as Sumatran tiger's (*Panthera tigris sumatrae*) social enrichment at Tambling Wildlife Nature Conservation Rescue Centre, Lampung, Indonesia. Biodiversitas 23: 55-61.* Tiger individuals are translocated to ex-situ conservations due to human-tiger conflicts and may express behavioral change (stereotypic) in captivity. Furthermore, medical check-up routines may cause injury and stress between tiger and operators under tough circumstances. Positive reinforcement conditioning (PRC) is a well-known method to minimize the risks on medical examination and as social enrichment. Therefore, the purposes of this research are (i) to examine PRC on tiger's blood sample collections and (ii) the correlation between physiological stress and tiger's stereotypic behavior (SB) through the neutrophil per lymphocyte ratio (N/L ratio) method. Four Sumatran tigers' (1 female, 3 males) behavior were observed using focal animal sampling at Tambling Wildlife Nature Conservation Rescue Centre, Lampung, Indonesia. Each baseline and post-enrichment tiger's behavioral observations were conducted for 1.920 minutes (1 male & 1 female) and 960 minutes (2 males). Then SB was categorized into low (<33,33%), intermediate (33,34-66,66%) or high (>66,67%). Blood collections (BC) were conducted twice (1st without PRC and 2nd with PRC) directly on the tiger after the behavior observation. Tiger's physiological stress during BC was analyzed with Wilcoxon. The correlation between physiological stress and SB was analyzed with Kruskal-Wallis. All tigers' N/L ratios were in normal value from this present research during both BC. The tigers did not show chronic stress as long as this research was conducted. PRC was significantly effective to reduce tiger physiological stress during BC (Z= -0,730, P= 0,465 < 0,05). All tigers showed low (<33,33%) pacing SB (without fur-plucking, self-biting, and self-mutilation behavior) both in baseline and post-enrichment. Any fur-plucking, self-biting & self-mutilation behaviors were not observed. Physiological stress was not correlated to SB (x^2= 3, P= 0,392 > 0,05). The PRC was an appropriate and effective tool to handle Sumatran tigers during BC. These tigers performed SB as a coping mechanism in the enclosure.

Keywords: N/L Ratio, PRC, social enrichment, stereotypic, Sumatran tiger

INTRODUCTION

Tiger population needs to be preserved, although it's facing continuous threats such as deforestation (Luskin et al. 2017; Poor et al. 2019), poaching (Risdianto et al. 2016), and human-animal conflict (Lubis et al. 2020). Tiger individuals are translocated to rescue centers or ex-situ conservations due to human-tiger conflicts. Tiger may express behavioral change (stereotypic) in captivity such as pacing, fur-plucking, self-biting, and self-mutilation (Stanton et al. 2015; Vaz et al. 2017). Those threats and behavioral changes also occur to Sumatran tiger (*Panthera tigris sumatrae,* Pocock 1929). Sumatran tiger as the only *Panthera tigris* subspecies left in Indonesia is well-known by its local names such as "Datuk", "Si Mbah" or "Kiai".

Handling a wild animal is risky. Injury between the non-domesticated animal and the operator during blood collection and injection on medical procedure may be present (Grandin 2000). Tiger's attack on its handler has been reported in a modern zoo (Tantius et al. 2016) and unregulated private zoo setting (Schiller et al. 2007). Stress causes abnormalities in medical procedures and diagnostic tests (Rodan 2010). Neutrophil/lymphocyte ratio (N/L ratio) can be applied to measure an animal's stress. Examination of the N/L ratio is a more practical and rapid method than hormonal assays to be performed in the field (Seltmann et al. 2020).

Many species have been successfully conditioned to cooperate with a veterinarian during medical examination (Grandin 2000). Tiger's standard hematological value is mentioned by others researchers (Shriavastav et al. 2012; Allwin et al. 2019). Positive reinforcement conditioning (PRC) using food "rewards" prepares the animal to voluntarily cooperate during blood collection/vein puncture (Coleman et al. 2008). PRC reduces stress during blood collection on antelope, bison (Grandin 2000), dog, and wolf (Vasconsellos et al. 2016) and applies to snow leopards (Broder et al. 2008) lions (Callealta et al. 2019)

and jaguar (Gracia et al. 2021). Negative reinforcement as "punishment" has been mentioned on the rat (Mayer et al. 2018; Hernandez-Lallement et al. 2020) and dog (China et al. 2020). In conditioning animals, positive reinforcement as a "reward" is more effective than "punishment" (China et al. 2020). "Punishment" impedes the learning process and intensifies anxiety (Rodan 2010). In addition, PRC works as a social enrichment on the tiger (Szokalski et al. 2012). There is no scientific publication about positive reinforcement conditioning in Sumatran tiger yet.

This research focuses on the Sumatran tiger's stereotypical behavior and physiological stress during blood collection. The lower stress level during blood collection may lead to more accurate medical examination and diagnoses. The purposes of this research are to (1) evaluate the effectiveness of PRC on Sumatran tigers' blood collection and (2) examine the correlation between physiological stress and stereotypic behavior through the N/L ratio. The results from this research may be valuable and applicable for rescue centers and ex-situ conservations as animal welfare's efforts.

MATERIALS AND METHODS

Ethical approval

Ethical clearance of this research was approved by the Ethics and Research Committee at the Faculty of Medicine Universitas Indonesia (No.20-01-0058), Indonesia. All procedures were conducted aseptically and with animal welfare considerations.

Study site and animals

This research was conducted in Tambling Wildlife Nature Conservation (Tambling) as a part of Bukit Barisan Selatan National Park, Indonesia. Data were collected from 4 adult Sumatran tigers (3 males & 1 female) at Tambling Rescue Centre. In this research, those tigers were named Tiger 1, Tiger 2, Tiger 3, and Tiger 4 (female). All tigers were born at Tambling Rescue Centre except for the female tiger (Tiger 4). The female tiger was translocated to Tambling Rescue Centre due to human-tiger conflict at Jambi. Age estimation on those tigers was: 8 years (Tiger 1), 9 years (Tiger 2 and Tiger 3), and 20 years (Tiger 4). Animal housing and veterinary care were followed Tambling's standard operating procedures and animal welfare principles.

Methods of sampling

Stereotypic behavior observation

Tigers' stereotypic behavior was observed using the focal continuous animal sampling method (Altmann 1974; Davis et al. 2018) within a radius of 10 m in the Tambling's Individual Cage. There was a visual barrier (fence) between the tiger and the observer. The tiger was observed twice (1st as baseline and 2nd as post-enrichment). The stereotypic behavior observation was conducted for almost 2.000 minutes. Tiger 1 & 2 were observed for 1.920 minutes, while Tiger 3 & 4 were observed for 960 minutes each baseline and post-enrichment behavior observation.

Any fur-plucking, self-biting, self-mutilation, and pacing were noted as stereotypic behavior (Mohapatra et al. 2014; Stanton et al. 2015; Vaz et al. 2017). One observer conducted stereotypic behavior observation for 4 days for each baseline and post-enrichment.

Positive reinforcement conditioning (PRC) and blood collection

Fresh chicken meats were chosen as a food reward on PRC (Callealta et al. 2019). The fresh chicken meat was parted into 12 pieces. The PRC was conducted in the mechanical restraint cage and only given to the tiger at the 2nd blood collection. Each tiger was granted a minimum of 3 pieces of chicken meat during the blood collection. The chicken meat was given to the tiger piece by piece during the blood collection. Then each tiger was granted 5 pieces of chicken meat after the blood collection.

Blood samples were collected with a mechanical restraint cage and without anesthesia induction. Standard blood collection was performed < 3 minutes with safety and animal welfare consideration (Allwin et al. 2019). Once the tiger was restrained, blood (0,5 mL) was collected through a coccygeal vein using a sterile syringe (3 mL) and needle (22 G). Blood smears were made directly from fresh blood after blood collection. The sterile object glass was given an eye drop of fresh blood (1st object glass). Then, another sterile object glass (2nd object glass) was attached within ±30° angle (Rebar et al. 2001; Theml et al. 2004) to the dropped fresh blood and pushed gently to make the blood smear. Methanol was used for blood smear fixation (2-3 minutes) (Bakhri 2018).

Blood smears staining

This research occurred on the field (outdoor condition). Blood smears fixation was done near the mechanical restraint cage. Furthermore, blood smears staining was conducted within the rescue center area but in a different location from the mechanical restraint cage. Therefore, location with room temperature, no direct sunlight, and rain/windy weather was preferred for blood smears staining to minimize the debris.

Blood smears were stained with Giemsa-Wright solution. After which, it was dipped into Wright's solution for 2 minutes. Blood smears were rinsed slowly using aquadest then dipped into 10% Giemsa solution for 15 minutes (Ardina and Roslinda 2018). After dipping in Giemsa solution, blood smears then rinsed slowly using aquadest. Blood smears were let to dry before N/L calculation under the microscope.

Neutrophil/lymphocyte ratio (N/L ratio) count

Differential leucocyte was observed under ×40-400 microscope magnification. Each neutrophil and lymphocyte were counted within 200 white blood cells under the microscope (Kerr 2002; Theml et al. 2004; Shiravastav et al. 2012). Then the counted neutrophils were manually divided by counted leucocytes to obtain the N/L ratio (Dhabhar et al. 1996; Stockman et al. 2011; Seltmann et al. 2020).

Duration (minutes) of stereotypical behavior recorded was transformed to a percentage (%) for a more straightforward interpretation. The mean of stereotypic behavior (baseline and post-enrichment) from each tiger was analyzed descriptively using Microsoft Excel® software. Stereotypic behavior was categorized as low (<33.33%), intermediate (33.34-66.66%) or high (>66.67%). Baseline (control) and post-enrichment (PRC) N/L ratios were analyzed with Wilcoxon to investigate physiological stress during blood collection. The correlation between physiological stress and stereotypic behavior was analyzed with Kruskal-Wallis. SPSS® 25 software was used for both Wilcoxon and Kruskal-Wallis analysis.

Figure 1. An individual tiger performed calmed behavior in the mechanical restraint cage

RESULTS AND DISCUSSION

Results

PRC effectiveness during blood collection as social enrichment

Tigers expressed calmed behavior within the mechanical cage (Figure 1), although they would not enter the mechanical restraint cage voluntarily. Therefore, the tiger must be lured with fresh chicken meat to enter the mechanical restraint cage by the Tambling's keeper. Blood collection was conducted after the tiger felt relaxed and calmed down in the mechanical restraint cage. Sudden noises and moves should be minimized during blood collection (<3 minutes) (Figure 2). Fresh chicken meats were given to distract the tiger's focus during the blood collection and as food reward after the blood collection. One operator gave parted chicken meat to the tiger by following the tiger's reactions and focuses. Each individual had a different speed when consuming the parted chicken meat.

Neutrophils and lymphocytes were observed under the microscope (Table 1, Figure 3). N/L ratio could be counted manually in the remote area. Statistical analysis showed that PRC significantly reduced physiological stress during blood sample collection (Z= -0,730, P= 0,465 < 0,05). Despite physiological stress reduction, all tigers expressed alert/cautious and aggressive behaviors towards human.

Stereotypic behavior and the correlation to physiological stress

All tigers performed low stereotypic (<33,33%) pacing behavior (Table 2, Figure 4). All tigers did not express any self-biting, self-mutilation, or fur-plucking behavior. Statistical analysis indicated that there was no correlation between physiological stress (N/L ratio) and stereotypic behavior ($x^2 = 3$, P= 0,392 > 0,05).

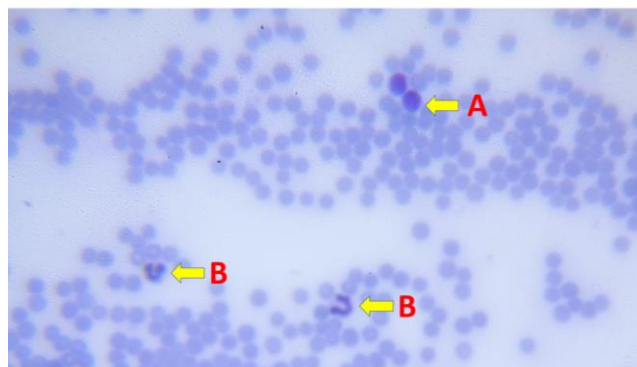**Figure 2**. Tiger's blood collection through a coccygeal vein in the mechanical restraint cage

Figure 3. Documentation of lymphocyte (A) and neutrophil (B) count under ×40-400 microscope magnification

Table 1. N/L Ratio of each tiger during blood collection

Tiger	Baseline (without PRC)	Post-enrichment (with PRC)
Tiger 1	1.52	2.03
Tiger 2	3.33	1.68
Tiger 3	0.83	1.57
Tiger 4	5.2	1.67

Table 2. Mean of tiger's stereotypic behavior

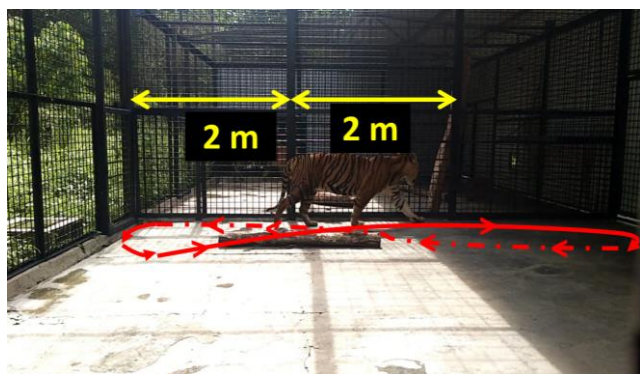| Tiger	Observation	Baseline	Post-enrichment
Tiger 1	1.920 minutes of	14.95%	12.29%
Tiger 2	observation	29.84%	13.07%
Tiger 3	960 minutes of	22.29%	5.63%
Tiger 4	observation	17.92%	16.04%

Figure 4. The route of the tiger's pacing behavior is almost similar to number "8" (showed by red lines or —)

Discussion

Effect of PRC on N/L ratio as a social enrichment

This present research showed tiger's N/L ratio differed for each individual after PRC. Descriptively, the tiger's N/L ratio was increased after PRC on Tiger 1 (from 1.52 to 2.03) and Tiger 3 (from 0.87 to 1.57). Meanwhile, Tiger 2 (from 3.33 to 1.68) and Tiger 4 (from 5.2 to 1.67) were reduced. All four tiger's N/L ratios were statistically analyzed with Wilcoxon and resulted from the that tiger's N/L ratio was significantly reduced (Z= -0,730, P= 0,465 < 0,05) with PRC. Its implied PRC could minimize distress during blood collection on the tiger. This is consistent with other research (Callealta et al. 2019), which successfully conducted PRC on African lions.

All Sumatran tigers' N/L ratio from this study were within the normal range of tigers' N/L ratio values. Even though Tiger 4's N/L ratio resulted in 5.2 at the 1st blood collection (without PRC), Tiger 4 was not in distress when this research was conducted. Naidenko and Alshinetskiy (2020) research large Felidae white blood cells including tigers' N/L ratio at zoos. The normal value of N/L ratio for large Felidae is 0-5.3. The N/L ratio value above 5.3 is considered as stress. They also explain that large Felidae has more neutrophils and monocytes than small Felidae. The number of lymphocytes is fewer in large Felidae than in small Felidae.

Abnormalities due to physiological stress on medical examination and diagnostic tests have been reported. Tachycardia, bradycardia, rapid breathing, pupil dilatation, hyperthermia, stress colitis, and urination may appear during the medical examination. While on the diagnostic test may appear on the complete blood count (platelet hypersensitivities, lymphocytosis, neutrophilia), blood chemistry (hyperglycemia, hypokalemia under epinephrin release), urine analysis (pH level alteration), and hypertension (Rodan 2010). Stress influences physiological conditions such as; immune system activation (Hellhammer et al. 2010; Narayan et al. 2017), metabolism, cardiovascular function, reproduction, behavior, and cognition (Narayan et al. 2017). Physiological changes appear as responses to the stressor/stimuli (Narayan et al. 2017). Stressor bothers physical's homeostatic then initiates flight or fight response. Flight or fight response involves autonomous activities and metabolic changes in an organism to adjust to the environment (Morgan and Tromborg 2007).

Stress and glucocorticoid treatment can increase neutrophils (neutrophilia). Glucocorticoid reduces lymphocytes (Sapolsky et al. 2000; Davis et al. 2008; Valenciano et al. 2010), eosinophils (Valenciano et al. 2010), basophils, macrophages, and monocytes (Sapolsky et al. 2000). N/L ratio escalates due to stressors (Schaefer et al. 1997; Davis et al. 2008). N/L ratio as stress measurement has been researched on cows (Stockmann et al. 2011), rhesus macaques (Lee et al. 2012), ebony leaf monkeys (Danafi et al. 2017; Titisari et al. 2019), and bats (Seltmann et al. 2017).

Species may adapt to cope (coping response) through stressing environment (stressor) (Hofer and East 1998; Sapolsky et al. 2000; Narayan et al. 2017) and anthropogenic disturbances (Hofer and East 1998). Stress is a component of a tiger's life cycle and is not inherited. Stress prepares individuals to perform hunting behavior, mating behavior, and feeding behavior (Narayan et al. 2017). Abiotic environmental stressor on wild animal has been recorded (Morgan and Tromborg 2007). In both environments, whether it's in captivity (ex-situ) or in the wild (in-situ), individuals will meet stressors in their environment. Not only from the anthropogenic disturbances, but also habitat factors will influence tigers' stress level. Malviya et al. (2018) study habitat factors such as canopy cover and shrub abundance that contributed to tiger's stress levels in India. Another factor contributing as a stressor in the wild is prey availability for tigers.

Visitors were restricted unless for conservation education and research considerations at the rescue center. Under that circumstances, blood collection (medical examination) with PRC on those tigers was categorized as social enrichment. This research supported scientific evidence regarding PRC as social enrichment for the tiger in line with Szokalski et al. (2012). The effect of the visitor on the animal behavior was reported from other researches (Sellinger and Ha 2005; Vidal et al. 2016; Suárez et al. 2017). Previous studies classified visitors as having negative interaction for animal welfare in zoos due to stress conditions for the animal. Contrary to Suárez et al. (2017), this research emphasized the positive human-animal interaction for animal welfare and aligned with Cole and Fraser (2018). Therefore, PRC was very beneficial in handling the Sumatran tiger during blood sample collection and as a tool to implement animal welfare in captivity.

The correlation between stereotypic behavior and physiological stress

Behavioral observation and physiological indicators were elaborated in this research. In line with other researches (Davis et al. 2008; Seltmann et al. 2020), this present research showed that the N/L ratio could be used as a physiological stress indicator in the field or remote area. Behavior observation was merely enough to measure stress response (Hofer and East 1998). Hematological value has essential information on effectively managing translocation and rehabilitation (Shanmugam et al. 2017).

The physiological parameter in this research represented no chronic stress after blood collection. All tigers also expressed feeding behavior after the blood was collected. According to previous research, the N/L ratio is more relevant to measuring acute stress conditions than chronic stress. Cortisol hormonal assay is more suitable for chronic stress than N/L ratio. Cortisol serum in the blood increases during animal restraint, while the N/L ratio is not influenced by blood collection (Swan and Hickman 2014).

Unexpectedly, there was no correlation between stereotypic behavior and physiological stress. A possible explanation was the tiger had reached a homeostatic state at the captivity and was found to be adapted to their new environment. As within the area of Bukit Barisan Selatan national park, Tambling Rescue Centre provided a natural enclosure for those tigers. Tigers were conditioned solitary in the Individual Cage (6 m $\times$ 6 m) and routinely transferred to the Habitual Cage (135 m $\times$ 57 m) with natural settings (every 1-2 weeks). This result highlighted other researches which stated that the animal expressed stereotypic behavior as a coping mechanism (Chosy et al. 2014). Tigers may live to co-exist in the human landscape (Carter et al. 2012; Struebig et al. 2018). The tiger may express pacing behavior due to external stimuli (captivity) and anticipation of a certain event (Krawczel et al. 2005). Contrary to previous research (Vaz et al. 2017), which recorded stereotypic behavior appearing due to stress conditions.

All tigers in this research performed aggressive behavior toward humans and tended to avoid humans. One phenomenon was observed within the overall observation in this research. Tiger 4 chooses to stalk and attack one keeper than live prey (boar) during feeding time. Fortunately, there were fences as barriers between the tiger and the keeper. The individual variation could not be separated for research despite the small sample size. From the previous study, individual variation has a biological function (Koolhas 2008). Coping mechanisms variation and personalities determine evolution competence (Sih et al. 2004; Bell 2007). Aggressive behavior is a crucial trait in surviving or adapting (Bell 2007).

According to previous research, lion species outsmart tiger species in problem-solving experiments. Social being has higher persistence than asocial being (Borrego and Gaines 2016). Social species (such as cats, humans, dogs, and horses) can learn social skills within species (species-specific skills). Social experiments within species are fundamental for social behavior development (Corwell-Davis et al. 2004; Ahola et al. 2017). Without social skills, an individual will fail to recognize and respond to the signals from another individual (greeting, submissive, dominance) and also increase stereotypic and aggression (Ahola et al. 2017). Newly introduced Sumatran tiger has been recorded to interact affiliative towards other tiger in Osnabrücker zoo (Schimmelphennig et al. 2017).

Despite the small sample size of this research (only four tigers), any information for the wild protected animal is valuable for conservation. Sample collections on this research were planned to reduce human-animal contact as minimum as possible. Other research already mentioned the animal welfare and ethic for the wild animal with 3Rs (Reduce, Refine and Replace) (Soulsbury et al. 2002).

Behavioral changes in free-ranging animals were recorded by other researchers (Kerley et al. 2002; Muhly et al. 2011; Smith et al. 2015; Suraci et al. 2019). Free-ranging species also expressed behavioral changes by force of anthropogenic disturbances. Human activities modify the habitats, which indirectly impacts animal behavior (Smith et al. 2015). Human activities potentially change the predator-prey interactions (Muhly et al. 2011). For example, the female Amur tiger is interested in the road made by humans. Those tigers used the road as a corridor within the tiger territorial-ranged. It increases the female tiger's mortality risks and reproduction failure (Kerley et al. 2002).

Although there was no correlation between physiological stress and stereotypic behavior in this research, stereotypic behavior should not be underestimated. Enrichment techniques are required to support animal welfare. Another study noted that novel enrichment techniques (Gomes et al. 2019; Clayton and Shrock 2020) and experience (Clayton and Shrock 2020) should be explored for tiger enrichment. Enrichment techniques must be suitable for species-specific behavior (AZA 2016). Therefore, any fur-plucking/self-biting/self-mutilation (another form of tiger's stereotypic behavior) could be prevented from being expressed by Sumatran tigers in captivity.

The outcome from this research is emphasizing PRC effectiveness during blood collection and minimizing physiological bias on medical examination on Sumatran tiger. It also provides social enrichment (as animal welfare implementation) for the Sumatran tiger. Therefore, PRC should be conducted in tigers' ex-situ conservation. In addition, N/L ratio is an efficient method to be performed on the field to measure the tigers' physiological stress. Sumatran tigers also may perform pacing behavior as a coping mechanism in captivity.

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