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Research article

Effect of brushing on heart rate variability in Malayan tapirs: Does brushing contribute toward alleviating puncture stress?

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Abstract

Many zoos conduct husbandry training with positive reinforcement to improve the care and welfare of wild animals. However, few studies have quantitatively examined whether stress occurs during invasive procedures. Brushing is known to encourage Malayan tapirs *Tapirus indicus* to lie down, helping with veterinary care, including preventive care. This study aimed to assess stress in Malayan tapirs during veterinary procedures using heart rate variability (HRV) analysis. In Experiment 1, the HRV index was tested by puncturing the tapirs without brushing and behavioural stress indicators were examined. Experiment 2 involved HRV measurement during husbandry training, including blood collection and brushing, to compare HRV changes before and after puncture. Experiment 3 examined the impact of brushing by comparing HRV across different postures, including during nighttime rest. The results showed that in Experiment 1, heart rate (HR) increased during and after puncture, matching the behavioural stress index, suggesting HRV analysis may be a useful stress evaluation tool. In Experiment 2, HR, HF, and LF/HF varied widely among individuals and days, with no clear negative impact of of puncture. Experiment 3 showed significant differences in HRV parameters (HR, HF, and LF/HF) across postures, suggesting that brushing may have a rewarding effect on the tapirs. This study allowed us to conduct the first HRV analysis of Malayan tapirs. In addition, the potential of reducing the stressful effects of invasive procedures by brushing was suggested.

Introduction

Zoos house various rare wild animals for conservation, education, research, and recreation (Hosey et al. 2009). However, the natural history of many of these animals is unknown, and many zoos make research efforts to establish breeding methods that are supported by scientific data and improve animal welfare. Husbandry training that facilitates various types of veterinary care, including blood collection without anesthesia and physical retention of animals, contributes to improvements in animal welfare (Irwin et al. 2013). However, very few studies have quantitatively examined whether training contributes to stress reduction during invasive procedures. Several methods exist to assess stress, including behavioural indices, endocrine indices (e.g., cortisol measurements), and autonomic nervous system function indices (Hosey et al. 2009). Since animals must be trained to keep still while blood collection is conducted, it is not possible to evaluate the changes in behaviour as an indicator. Although glucocorticoids such as cortisol are useful for estimating physiological stress levels, the time lag between the short-term stress load and the increase in their levels in the blood pose a problem for measurement (Hernandez et al. 2014). Because the duration of invasive procedures such as blood collection is very short, it is better to use methodologies that can capture immediate stress responses, such as heart rate (HR) and heart rate variability (HRV). HR and HRV analyses have been widely used in humans, farm animals, and companion animals to assess various conditions, including stress and fatigue (Bowman et al. 2017; Järvelin-Pasanen et al. 2018; Kovács et al. 2014). HRV analysis allows a more detailed interpretation of autonomic nervous activity compared to HR analysis. For example, the frequency domain methods used in HRV analysis provide insights into the interplay between the activities of the sympathetic and parasympathetic nervous systems (Hagen et al. 2005). The frequency band of the respiratory rate determines the HRV band pattern. High frequency bands (HF) are primarily controlled by the cardiac parasympathetic nervous system (the parasympathetic nervous system is active during relaxation and rest and plays a role in stabilizing the heartbeat), while low frequency bands (LF) are controlled by both the sympathetic and parasympathetic nervous systems. The sympathetic nervous system is involved in physical activity and stress responses and may contribute to increases in HR and blood pressure. The LF to HF ratio (LF/HF) represents the balance between sympathetic and parasympathetic activity (Hagen et al. 2005; Billman 2013). In this study, therefore, we considered the assessment of autonomic tone to be suitable as a short-term stress indicator.

At the Chiba Zoological Park, Japan, Malayan tapirs *Tapirus indicus* are subjected to husbandry training for medical examinations and research that aims to elucidate the dynamics of sex hormones for breeding. Blood sampling was performed regularly by a veterinarian to achieve the aforementioned purposes. Malayan tapirs have the unique characteristic of being able to be immobilized in a prone position by brushing (Kusuda et al. 2007; Rose and Roffe 2013). However, the changes in the autonomic nervous system in Malayan tapirs during unsecured blood collection and the evaluation of stress during puncture have not yet been investigated. In this study, we examined the validity of the HRV index as a method for evaluating stress in Malayan tapirs and investigated changes in the HRV index during blood collection with brushing, aiming to determine whether training and brushing could alleviate puncture-related stress.

Materials and Methods

Study subjects

The study focused on three Malayan tapirs housed at Chiba Zoological Park, Chiba, Japan: a male (Yumeta) and two females (Yumeta's mother Yukimi and Sako; Table 1). The exhibit area for the Malayan tapirs consisted of three separate areas, with one animal housed alone in each area.

The animals were rotated daily, except during the estrus season, when Yumeta and Sako were housed together in one exhibit area for breeding. Estrus occurred at intervals of approximately 29 to 31 days, and each estrus lasted 3 to 4 days. The experiments were conducted during the nonestrus season. The animals were housed in outdoor enclosures from 09:00 to 16:30 between April and October and from 09:30 to 16:00 between November and March. From 16:00 to the next morning, the animals were individually housed in an indoor enclosure constructed using reinforced concrete. During morning feeding, ~6 kg of roughage was provided in the enclosures, and ~13 kg was provided in the house for the evening feed.

Training

Blood collection training began in May 2014 and was successful for Yukimi in July 2014, Sako in September 2014, and Yumeta in July 2015. The animals were housed in the tapir house in the evening and, approximately twice a week, underwent a gradual increase in the intensity of stimulation, starting with brushing and lying down (Figure 1), progressing to finger and toothpick stimulation, and eventually leading to blood collection. From January 2016 to April 2018, blood collections were conducted once a week to monitor sex hormone dynamics in the females (Yukimi and Sako). Yumeta also underwent blood collection twice a year for health assessments. Additionally, all three tapirs received training for echographic examinations and oral condition checks, which occurred approximately three times a week. For echographic examination, the actual machine was only used two times a year for health checkups. However, training was conducted five days in a week using a dummy to ensure that it was carried out smoothly. Specifically, we desensitized the animal to the echo probe by using a showerhead as a dummy. These training sessions were conducted by keepers. During training, veterinarians were occasionally present for oral checks and blood collection. Operant positive conditioning techniques were used in the training sessions, and all sessions involved brushing. For the oral condition check, a fruit was used as a primary reinforcer and a whistle as a secondary reinforcer to reinforce mouth-opening behaviour. The oral check aimed to detect oral and dental abnormalities at an early stage. Given that Malayan tapirs lie down when brushing, training for blood sampling and ultrasound examinations can be performed in this position (Kusuda et al. 2007; Rose and Roffe 2013). By

Table 1. Information on surveyed individuals

Name	Yukimi	Sako	Yumeta
Sex	Female	Female	Male
Birth	14 July 1991	12 December 2007	26 July 2006
When to start blood collection training	May 2014	May 2014	May 2014
When the first successful blood collection	July 2014	September 2014	July 2015
Experiments on offer (*)	i,ii	ii,iii	ii,iii
Number of attempts	i: once	ii: twice	ii: twice
	ii: twice	iii: once	iii: once

(*) i, ii, and iii each represent the number of the experiment in which Malayan tapir participated, each of which includes the following:
(i) Validation of the Heart Rate Variability (HRV) index as a method of stress assessment in Malayan tapirs, ii, and iii
(ii) Changes in HRV indices before and after puncture during brushing
(iii) HRV indices during brushing and at night.

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Figure 1. A Malayan tapir lying on its side with a deck brush. Photo taken by Yasuhiro Hori at the Chiba Zoological Park in 2016. The person brushing the tapir was the author.

Figure 2. A sensor and horse belt on a Malayan tapir (Sako).

continuously brushing the Malayan tapir's back and buttocks with a deck brush, tapirs lie down (Figure 1), and while they were in that position, puncture stimulation and a dummy echo machine were applied to desensitize it to the actual veterinary treatment. In addition, considering that the body and limbs were not restrained, the animals could return from the prone position to the standing position at any time if a negative effect was present.

This study was conducted as part of the Collaborative Research Program of the Wildlife Research Center, Kyoto University, 2017-A-15. The experimental design was planned in accordance with the ethical code of the research center and the Japanese Association of Zoos and Aquariums, and the design complied with the Japanese Animal Protection and Management Act. The study used the opportunity of routine husbandry and management, and no blood samples or intramuscular injections were taken solely for research purposes.

Materials

Malayan tapirs were immobilized by using a deck brush (TAKASAGO). This brush was also used for all training and experiments. Behaviours were recorded using a camera (YKS-HDW3201R, YKmusen), recorder (RD-HF-0431-E2, YK Radio), and monitor (LEDDTV1326-10J, COBY). A V800 POLAR, which consists of a sensor with electrodes (hereafter referred to as "sensor") and a wristwatch receiver, was used to measure HR. A horse belt (EQUINE H10 POLAR) and keratin cream (FUKUDADENSHI OJE-01D) were used to secure the sensor to the animal's body. Syringes (10cc TERUMO) and needles (21G TERUMO) were used for blood collection.

Methods

HRV analysis was performed by securing the sensor to the left lateral side of the subject's body using a horse belt during the time the Malayan tapir was in the house (Figure 2). The area where the sensor was fixed was coated with keratin cream to cover an area ~10 cm in diameter to improve the sensor transmission. The sensor was attached to the tapirs after they had been trained to wrap a string around their body using dried bottle gourd fruit *Lagenaria siceraria* var. *hispida* and edible dried gourd shavings. A wristwatch receiver was worn on the left arm by the keeper conducting the brushing. All nonveterinary activities related to Malayan tapirs, such as sensor attachment, brushing, and training, were performed by the same keeper, and only veterinary care was provided by the veterinarians.

Experiment 1: Validation of the HRV index as a method of stress assessment in Malayan tapirs

We analyzed the HRV of a Malayan tapir (Yukimi) during puncture procedures conducted without brushing. HRV analysis was conducted on an individual considered by a vet to require medication via intramuscular injection. Muscle injection training by brushing is difficult and has not been routinely performed, so the animals were not acclimated. The drug injected was enrofloxacin, which has no effect on cardiac function (Durgut et al. 2016).The behavioural assessment consisted of Interval focal sampling every 10 s for ~36 min, from 5 min before the muscle injection until the HR sensor was removed. Behaviour was determined according to Table 2.

Experiment 2: Changes in HRV indices before and after puncture during brushing

HRV analysis was conducted during husbandry training sessions, which included blood collection and brushing. Malayan tapirs equipped with an HR sensor were brushed and placed in a supine position. Approximately 3 min after the Malayan tapirs were placed in a supine position, the veterinarian entered the room to collect blood samples. Although no time limit was set, the blood collection process took around 3 min. The needle puncture time was ~30 sec. After blood collection was completed, the keeper continued brushing for ~3 min, while the vet left the room. The tapir was supine at all times while data on HRV indices were collected.

Experiment 3: HRV indices during brushing and at night.

Differences in HRV indices were examined between the condition of brushing and that of supine, prone, and standing still during

Table 2. Ethogram of stress behaviours and resting postures of tapirs.

Category	Behaviour	Definition
Stress behaviour	Escape	Keep distance from the object (*)
	Pawing	Scratch the ground with one's forelimbs (*)
	Heavy breathing	Breathing causes the back to rise and fall more than normal (*)
Posture	Standing	Standing position on all four limbs (**)
	Prone	Prone posture with all four limbs bent and chest up (**)
	Supine	Throwing out all four limbs (**)

(*) Definitions of behaviour were modified from ethograms of Young et al. (2012).
(**) Definitions of behaviour were modified from ethograms of Satou et al. (2011) .

the night. For brushing, HRV was recorded for at least 10 min, generally between 16:00 and 17:30 after the animals were moved from the outdoor to the indoor enclosure. During the brushing for experiment 3, tapirs were only brushed and no other husbandry procedures were performed (e.g., blood collection). Nocturnal behaviours were recorded by a camera and analyzed later. Behavioural assessment was based on body posture from the videos taken (Table 2).

Data analysis

Kubios HRV premium (3.0.2) was used to analyze the data. For each 2-min interval, HRV parameters were calculated using time and frequency domain analyses. We calculated the mean HR in the time domain analysis. In the frequency domain analysis, the normalized power of the HF band and the ratio of the normalized LF:HF ratio were calculated using the fast Fourier transformation algorithm of the power spectrum analysis. The LF range was set as 0.04–0.10 Hz, and the HF range was set as 0.10–0.40 Hz based on the respiratory rate of tapirs (6–10 times per min) calculated on-site. Automatic correction in the software was applied to correct the artifact. Given that a 102.4 sec time window was used to study the HRV in cattle (Kojima et al. 2024), a 2 min time window was set for this study based on HR of 40–80 bpm in tapirs. This ensured a sufficient resampling could be secured. A previous study in humans has reported that a minimum 40 sec time window is sufficient for acute pain assessment (Jiang et al. 2017). The duration of injection, blood collection, and related brushing was relatively short (up to 30 sec).

For the analyses of experiment 1, changes were visually described using the parameters described above before and after the injection without brushing. For the analyses of experiment 2, the moving average of HR, HF, LF were calculated, and LF/HF shifted by 10 sec three times for each condition (before, during, and after the injection). For example, if the injection started at 12:03:15, HF was calculated in the time range of 12:01:15–12:03:15, 12:01:05–12:03:05, and 12:00:55–12:02:55 for the condition "before injection" and calculated 12:03:15–12:05:15, 12:03:25–12:05:25, and 12:03:35–12:05:35 for the condition "during injection." The average of these three values was used to visualize and compare the differences. For the analyses of experiment 3, the period when the HR measurements were continuously stable were selected and divided the night-time data into three categories based on the postures of the tapirs: prone, supine, and standing. HR, HF, and LF/HF were sampled from two bouts of each sleeping posture

Table 3. The results of statistical analyses comparing HRV parameters across different postures.

Parameters	Comparison	Sako (W, P value)	Yumeta (W, P value)
HR	Brushing vs. Supine	W=315, P<0.001	W=159, P<0.001
HR	Brushing vs. Prone	W=151, P=0.0093	W=175, P<0.001
HR	Brushing vs. Standing	W=29, P=0.86	W=15, P=0.52
HF	Brushing vs. Supine	W=25, P<0.001	W=0, P<0.001
HF	Brushing vs. Prone	W=21, P<0.001	W=9, P<0.001
HF	Brushing vs. Standing	W=30, P=0.78	W=30, P=0.17
LF/HF	Brushing vs. Prone	W=290, P<0.001	W=117, P=0.11
LF/HF	Brushing vs. Prone	W=169, P<0.001	W=106, P=0.70
LF/HF	Brushing vs. Standing	W=5, P=0.0076	W=0, P=0.0016

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(prone and supine). Data was excluded when tapirs changed their postures. As each tapir showed different durations for each posture, the duration of the data collection differed among individuals as follows: a total of 83 points (2 min intervals) for Yumeta (10 mins during brushing and 156 mins during the night) and 73 points (18 mins during brushing and 128 mins during the night) for Sako. Tapirs sometimes changed their postures during the night; mostly they repeated prone and supine postures in turn.

HR, HF, and LF/HF during brushing were compared with those during the night using Kruskal–Wallis tests to compare HR, HF, and LF/HF among different postures with those during brushing. Significant differences were tested using post hoc Mann–Whitney U tests to compare the differences in each parameter between postures. Bonferroni corrections were applied for multiple testing. We did not use any statistical analyses for Experiments 1 and 2 considering the limited sample size. Given the limited sample size only descriptive data are provided for Experiments 1 and 2. Analyses were performed in R software version 4.1.2 (R Development Core Team 2021).

Results

Experiment 1

HR of Yukimi was found to increase at the time of puncture and immediately after the puncture. Behaviours also changed as the animal walked around the room immediately after the muscle

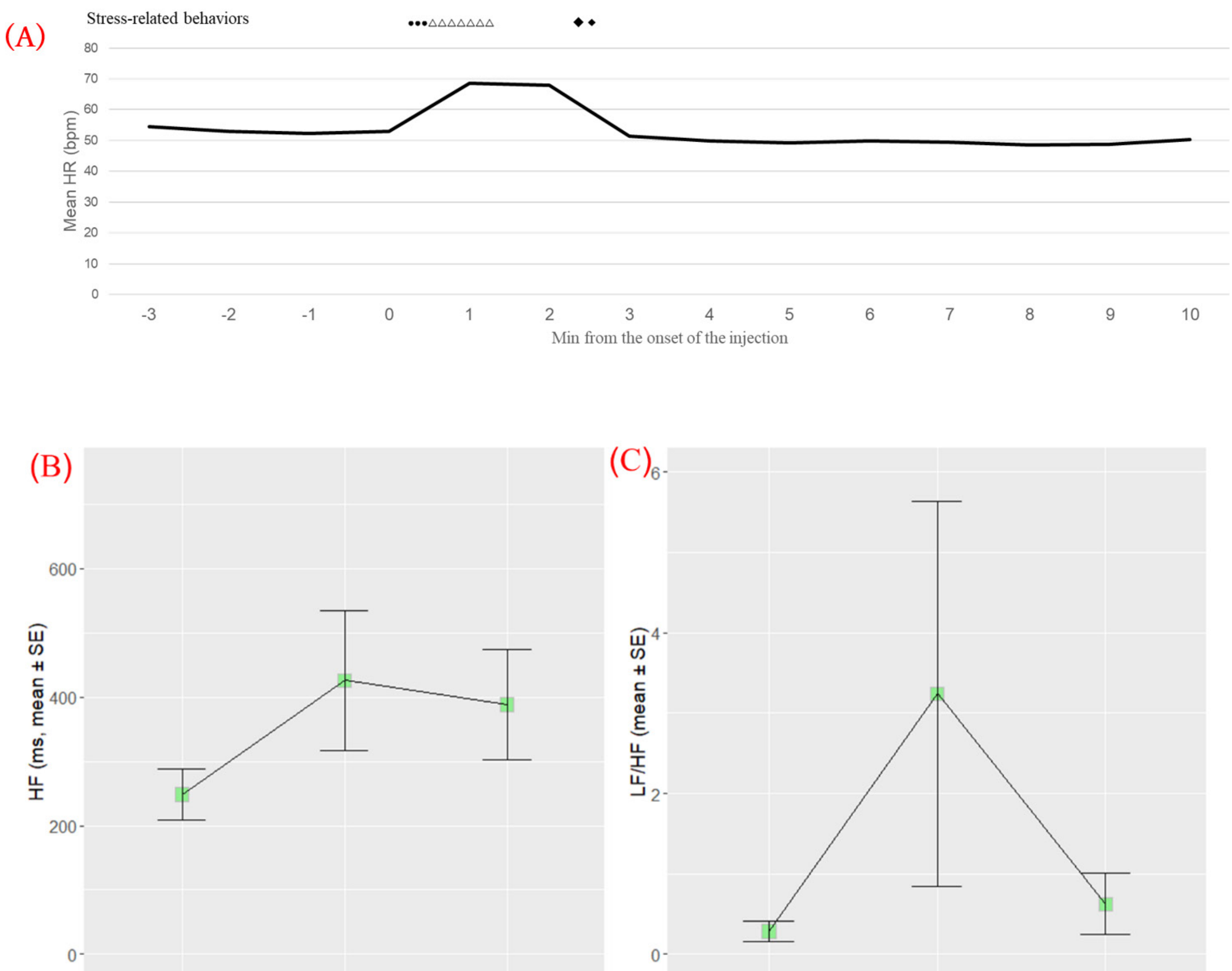

Figure 3. Figure 3.(A) Results of behavioral observations and heart rate (HR) changed according to Table 2, with muscle injection as 0 min.
●: escape, triangle: heavy breathing, diamond: pawing. (B) High Frequency (HF) changes before, during, and after intramuscular injection were shown. (C) Low frequency to High frequency ratio (LF/HF) changed from before, during, and after intramuscular injection.

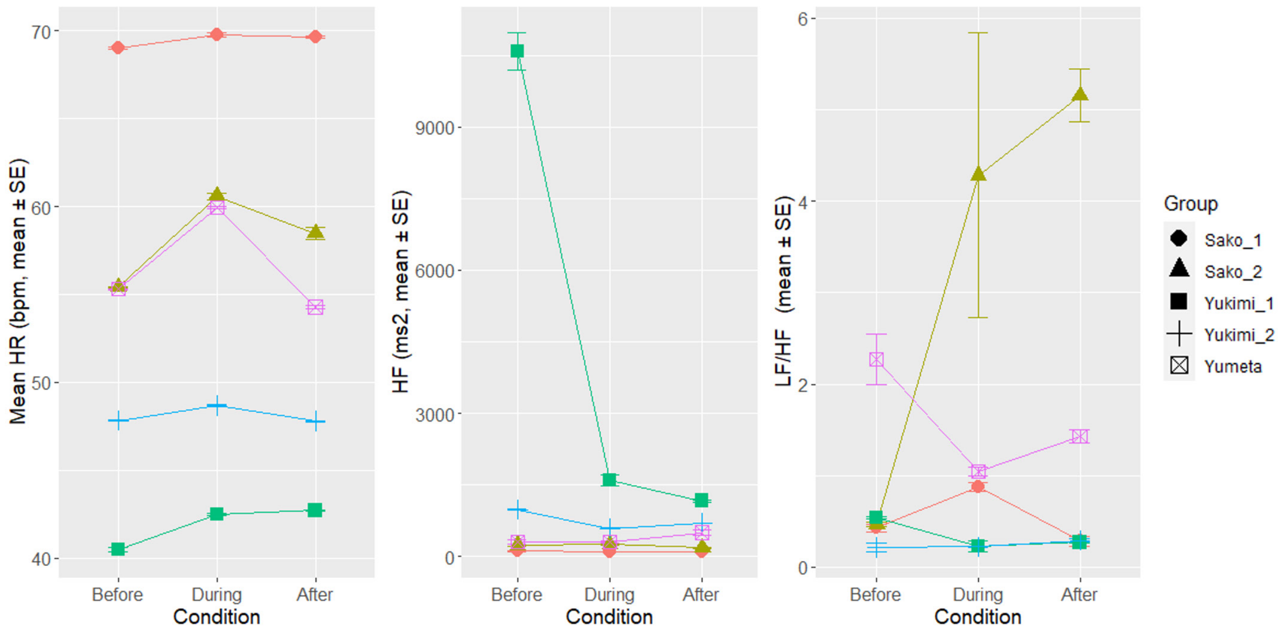

Figure 4. HR, HF, and LF/HF changed from before, during, and after puncture for blood collection while brushing.

injection and breathed more heavily while Yukimi stood still before the puncture. Furthermore, pawing was observed (Figure 3A). Meanwhile, HF and LF/HF increased Figures 3(B) and 3(C).

Experiment 2

The HR, HF, and LF/HF varied widely from individual to individual and day to day and we could not find a consistent trend (Figure 4). For example, Mean HR of Yumeta increased during the puncture, while LF/HF decreased and HF was almost the same across the conditions. In contrast, Sako did not show changes in HR and HF across the conditions while LF/HF increased during the puncture.

Experiment 3

Significant differences in HR (Figure 5A: Sako, X^2=35.8, df=3, P<0.001; Yumeta, X^2=47.6, df=3, P<0.001), HF (Figure 5B: Sako, X^2=30.6, df=3, P<0.001; Yumeta, X^2=56.8, df=3, P<0.001), and LF/HF (Figure 5C: Sako, X^2=27.4, df=3, P<0.001; Yumeta, X^2=21.8, df=3, P<0.001) were observed across postures in both individuals.

Specifically, HR during brushing was significantly higher than that during the supine or prone positions, but did not differ significantly from that during standing (Table 3, Figure 5A). HF during brushing was significantly higher than that in the supine and prone positions (Table 3, Figure 5B), but did not differ significantly from that during standing. For LF/HF, brushing values were significantly different from those in the supine position for Sako but not for Yumeta, and similarly for the prone position. The LF/HF during brushing was also significantly lower than that during standing (Table 3, Figure 5C).

Discussion

To the best of our knowledge, this is the first attempt to analyze HRV in Malayan tapirs. In experiment 1, the HR, HF and LF/HF increased immediately after muscle injection with puncture without brushing, which was considered to be stressful for Malayan tapirs. This suggests the sympathetic nervous system, which is activated by excitement and stress (Kojima et al. 2024), was dominant. Behaviours also changed immediately after muscle injection. The behaviours observed after puncture, such as pawing and heavy breathing, were similar to those observed in horses *Equus ferus caballus* during stressful situations (Young et al. 2012). Similar HRV dynamics were exhibited in management processes that were considered stressful, such as transport in horses (Ishizaka et al. 2017) and rectal examination in cattle (Kovács et al. 2014). The accompanying behaviours, which were frequently associated with stress, also suggest that HRV analysis is a useful method for assessing stress in Malayan tapirs.

In experiment 2, the effect of brushing on puncture stress was inconsistent across individuals and trials, as were the values of HR, HF, and LF/HF. Although it was difficult to generalize due to variables such as the veterinarian's blood collection technique, there was no more obvious negative effect than without brushing in most of the time.

In zoo animal husbandry, one of the goals of training is to immobilize animals without physical and chemical restraint and to improve the quality of routine husbandry and veterinary care (Fernandez 2022). Numerous cases, including the immobilization

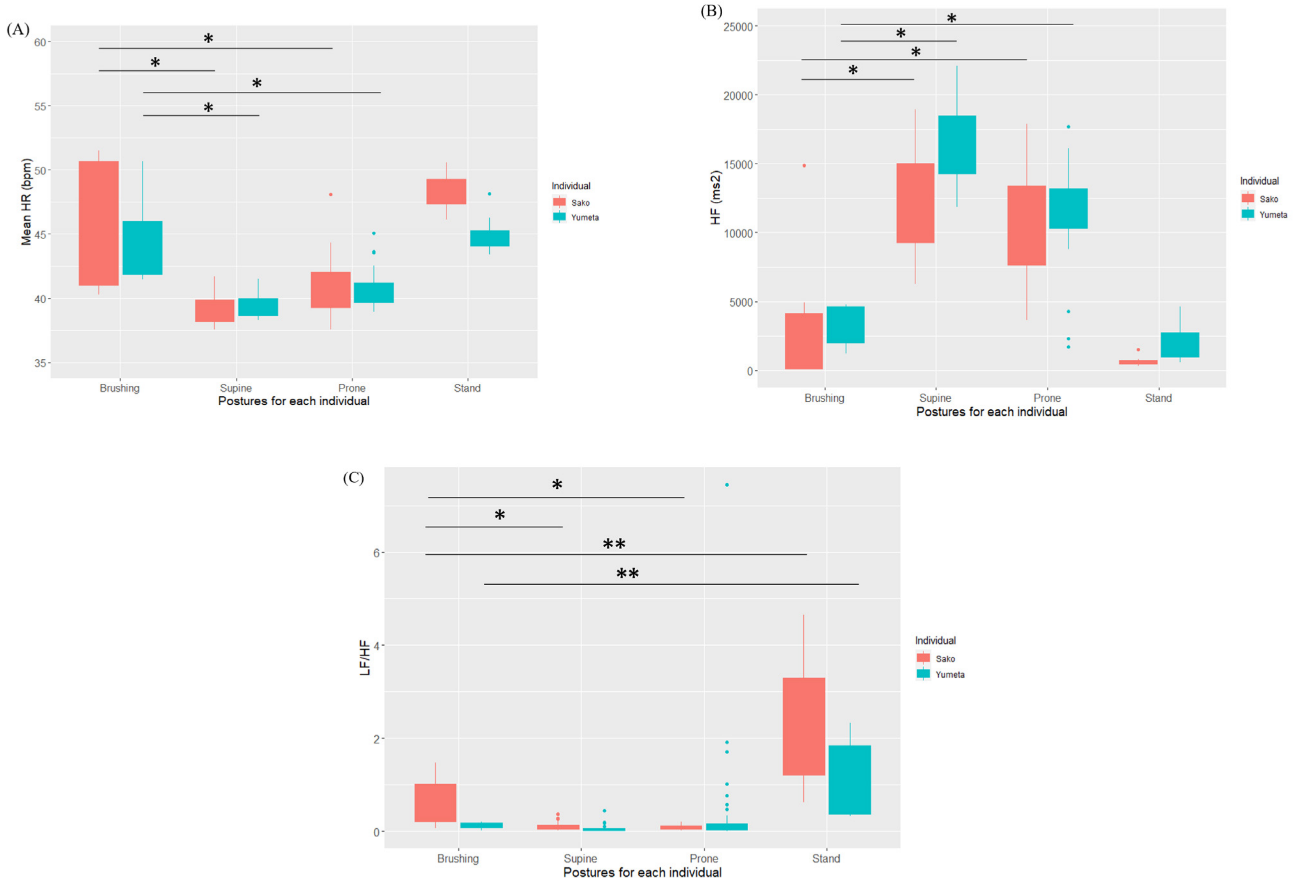

Figure 5.(A) Changes in HR during brushing and in each posture (standing, prone, and supine) at night. In both tapirs, HR during brushing was higher than that while supine or in the prone position. (*: P<0.001 Kruskal–Wallis tests). (B) Changes in HF during brushing and in each posture (standing, prone, and supine) at night. In both tapirs, HF during brushing was higher than that while supine or in the prone position. (*: P<0.001 Kruskal–Wallis tests). (C) Changes in LF/HF during brushing and in each posture (standing, prone, and supine) at night. In Sako, LF/HF during brushing were higher than that while supine or in the prone position (*: P<0.001 Kruskal–Wallis tests).In both tapirs, The LF/HF during brushing was lower than that while standing position (**: P<0.01 Kruskal–Wallis tests).

of nyala *Tragelaphus angasi* and bongo *Tragelaphus eurycerus* at Denver Zoo, have demonstrated that the use of training techniques without anesthesia or squeeze cages to inject insulin (Grandin et al. 1995). Some cases, including one in which insulin injections were given to the bongo (Grandin et al., 1995), have demonstrated immobilization using training techniques without anesthesia or squeeze cages. Immobilizing animals without physical and chemical restraints increases safety for both animals and caretakers. Appropriate veterinary care can be expected to prevent or speed recovery from conditions that impair animal welfare, such as disease and injury, and to improve the physical and mental condition of animals by controlling food intake, hoof care, and other physical maintenance through daily care and management. The fact that we were able to analyze the HRV during husbandry training and specifically investigate its effects in this experiment further reinforces the importance of training in zoo animal husbandry.

For experiment 3, the indices of HRV in the supine and prone positions tended to be similar to those reported in cattle (Frondelius et al. 2015). Brushing showed an index of HRV that was closer to that of standing compared to that when supine or prone. Since previous studies in domestic animals such as horses (Feh and Mazières 1993) and sheep (Tamioso et al. 2018) have reported the stress-relieving effects of brushing, we initially expected that HRV during brushing would be parasympathetic-dominant, as during sleep or lying down, but this was not the case in the present experiment. However, there may be a limitation in that this comparison was made between brushing and sleeping at night. In a previous study, brushing was performed in the awake state while standing up, making a comparison between awake conditions. In addition, the comparison of with and without brushing was made during the stressful environment of being separated from one's mate (Reefmann et al. 2009). In comparison, the present study was originally conducted on animals kept alone, and brushing was not performed under stressful conditions. Therefore, the conditions in the previous study were different from those in this study, making it difficult to directly compare the results. Another limitation in the analysis is that in the present study, we could not clearly evaluate whether or not the animals were sleeping from the video. Ideally, it would be better to experimentally create a situation when tapirs lie down without brushing and without sleeping for comparison. However, Malayan tapirs lie down when brushed and immediately return from lying to stand when brushing is stopped. Therefore, it was not possible to create such situation.

The HRV index during brushing was closer to the standing state and different from that at the supine and prone positions. However, brushing may function as a positive reinforcer for tapirs. The behavioural response of tapirs lying down when brushed is well known by keepers and is used in various zoos for blood collection (Kusuda et al. 2007; Rose and Roffe 2013) and other health management procedures (Pollock and Ramsay 2003). However, this behavioural response has not been investigated in detail. In sheep, brushing decreases the HR, which is consistent with behavioural indicators such as few ear posture changes, low proportions of forward ear postures, low relative eye aperture and a low variance in body surface humidity (Reefmann et al. 2009). By contrast, high brush use under stressful situations, such as isolation and lameness, in bovines had no effect on the HR (Mandel et al. 2018; 2019). Therefore, although the effects of brushing on HR vary among species, brush use may have a rewarding value for some animals. Considering that the Malayan tapirs used in this experiment were brushed after feeding, the brush itself was a reinforcer, as no other component of positive reinforcement occurred other than brushing. In addition, given that the body and limbs were not restrained, the animals could return from the prone position to the standing position at any time if a negative effect was present. Thus, sympathetic dominance may be viewed as positive excitement. A previous study in canines has shown that such dominance may reflect an increase in positive arousal while experiencing pleasant emotional stimuli, such as the presentation of food or grooming from a familiar human (Zupan et al. 2016). However, as we could not exclude the other possibilities (e.g., negative arousal), further research is necessary to elucidate this phenomenon. Furthermore, one of the tapirs was elderly, and old animals lose the elasticity of their blood vessel walls, and their blood vessels become stiff (O'Rourke and Hashimoto 2007). Although an engorged blood vessel was found after bloodletting, the vessel often moved when punctured, making it difficult to insert the needle. Therefore, veterinarians had to stretch the skin in the area where blood was collected or puncture the blood vessels more forcefully. This procedure may have led to the decrease in HF in Experiment 2. Moreover, one of the tapirs had less brushing experience than the other two. This factor may have prevented the positive effect of adequate brushing. Therefore, additional studies with detailed condition comparisons are necessary to clarify these variables.

A previous study in humans has reported that the minimum 40sec time window was sufficient for acute pain assessment (Jiang et al. 2017). In this study, the duration of injection, blood collection, and related brushing was relatively short; thus, we set up the 2 min time window for HRV analyses based on the respiratory rate of tapirs assessed on the site. As this study was the first trial in Malaysian tapir, further studies are necessary to determine the methodological details, including the appropriate time window.

This study allowed us to conduct the first HRV analysis of Malayan tapirs. In addition, the potential of reducing the effects of invasive procedures by brushing was suggested. Future work will require an increase in the number of samples and comparisons among various conditions.

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