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# Detection of HSP70 in Working Horses Using ELISA on Sumbawa Island

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**Abstract:** Heat shock protein 70 also known as HSP70 is a protein that regulates in response to stress factors such as heat, exercise, and others. The current research work used enzyme-linked immunosorbent assay (ELISA) to measure HSP70 levels in working horses on Sumbawa Island. Blood was taken through jugular venipuncture whereas saliva was collected non-invasively via cotton swab, both were analyzed with ELISA. The results indicated different expressions of HSP70; saliva shows a significantly higher level of HSP70 (52.07 ± 25.25 ng/mL) than serum (43.71 ± 34.83 ng/mL) from the same animal. The observed differences might indicate a better representation of acute stress by saliva due to physiological rapid turnover compared to serum, which reflects an integration of cumulative stress exposure. Our finding thus proves that double monitoring of both biological matrices offers complementary information in assessing stress in working horses while taking salivary HSP70 into account as especially promising for the in-field welfare monitoring due to its non-invasive collection and sensitivity to immediate stress conditions.
**Keywords**: HSP70; ELISA; working horses; Sumbawa Island; heat stress

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## INTRODUCTION

Sumbawa Island, located in the West Nusa Tenggara Province of Indonesia, is an island known for its large population of horses (*Equus caballus*). The horse population in Sumbawa Regency reached 14,378 heads by 2020 (BPS, 2024). The community on Sumbawa Island has utilized horses as working animals, making it essential to pay attention to their welfare. Working horses are potentially more susceptible to stress, especially since Sumbawa Island has a tropical climate that can trigger heat stress and subsequently affect welfare. The welfare status of working animals with respect to human-animal interactions has been a worldwide concern, as it can induce suffering in animals and is ingestly associated with people's welfare. The World Organisation for Animal Health, which developed animal welfare standards is a reliable source of welfare due to working animals' function, particularly in pulling animals, as income generating activities (OIE, 2016).

Animal welfare issues are often encountered in developing countries with tropical climates, which may face challenges in providing feed and dealing with hot weather. Heat stress and nutritional deficiencies have been identified as factors that significantly

affect the welfare, health, production and reproduction of animals worldwide, especially in the tropics (Niyas et al., 2017). The range of ambient temperatures known as the thermoneutral zone is where homeothermic animals maintain their core body temperature by balancing heat production and loss (Renaudeau et al., 2012; Santos et al., 2022). Thermal stress happens when ambient variables rise above the threshold of the thermoneutral zone, triggering physiological and behavioral reactions to maintain homeostasis (Bernabucci et al., 2010; Santos et al., 2022). These challenges manifest in measurable indicators: research on horses in Chile has shown disturbances in equine welfare, with a significant correlation between poor body condition and lack of deworming as well as between hoof deformities and reduced frequency of horseshoeing (Luna et al., 2017). A study on working horses in Indonesia, specifically in Gili Trawangan, West Nusa Tenggara Province, indicated poor body condition, with body condition scores of 2 (31.6%) and 1 (7.9%) among 38 horses examined (Pinsky et al., 2019).

Assessments of the state of animal welfare have been made using various methods related to the Five Freedoms. The five freedoms include: (1) Freedom from hunger or thirst; (2) Freedom from discomfort; (3) Freedom from pain, injury or illness; (4) Freedom to express normal behavior; (5) Freedom from fear and distress (Webster, 2022). Common measurements of stress in animals include heart rate frequency, blood or saliva cortisol levels, eye temperature, and various parameters of physiological behavioral changes (König et al., 2017).

Research on animal welfare using clinical parameters and various physiological behavioral change parameters, as previously conducted on working horses, will require special expertise and long-term training with various parameters from different regions. Therefore, molecular-based research is needed. Theoretically, horses with poor welfare in tropical regions like Sumbawa Island will initially experience cellular stress triggered by heat stress. Horse cells experiencing heat stress will express protective proteins against heat stress known as heat shock proteins (HSP). HSPs are involved in protecting cells from heat stress by preventing cellular protein denaturation (Feder & Hofmann, 1999). The most abundant and conserved HSP when cells experience heat stress is HSP with a molecular mass of 70 kDa, referred to as HSP70 (Hassan et al., 2019).

Based on the presence of HSP in cells experiencing stress, recent advances in stress physiology have identified HSP70 as a sensitive molecular marker that bridges observed welfare deficits with measurable cellular responses, research is needed to detect the presence of HSP70 in working horses on Sumbawa Island as preliminary data indicating stress, which will be useful in addressing the welfare issues of working horses on Sumbawa Island. As demonstrated in tropical working equids, HSP70 expression correlates strongly with both environmental heat load and workload intensity, providing a quantifiable link between external stressors and internal physiological strain. Working horses with poor welfare status will have reduced immune function, leading to suboptimal work performance because their energy is insufficient to cope with stressors, thereby disrupting their ability to maintain homeostasis, which can result in illness and death.

## METHOD

### Animals and Experimental Conditions

In this research study, there were 15 working horses from Sumbawa Island, Indonesia, which were actively used for transport and work-related projects. The sample size was determined based on: (1) typical herd sizes of working horses

maintained by individual owners in Sumbawa (average 3-5 horses per household), (2) logistical constraints of sample collection under field conditions. The horses were directly exposed to environmental temperatures of 30-33 °C, which is consistent with the tropical climate found in Sumbawa.

**Experimental Design**

The research utilized a completely randomized design (CRD) the horse was the experimental unit. HSP70 was measured in blood serum and saliva samples. Sample collection was made only once per horse once their normal working routine was completed. Sample collection was completed at several farm locations with more than five working horses - subjects were selected utilizing simple random sampling with a random number table. All sampled horses were regularly exposed to environmental heat (30–33°C), average working duration was 6–8 hours per day, therefore when HSP70 was measured, we made sure it was representative of stress due to prolonged heat exposure and physical exertion. The primary variables analyzed were:

1) HSP70 concentration in serum (ng/mL)
2) HSP70 concentration in saliva (ng/mL)
3) Environmental temperature during sampling (°C)
4) Work duration and intensity (hours/day, distance covered, and load carried), Based on owner reports and researcher verification, all study horses were routinely used for transportation (carrying 4-6 passengers per trip) and agricultural product transport (with typical loads of 80-120 kg), working an average of 7.2 ± 1.4 hours daily under tropical conditions (ambient temperature 30-33°C, relative humidity 65-80%). Work parameters including duration, load weight, and travel routes were documented through daily owner logs and spot-checked by researchers.

The sampling was performed under identical conditions across all horses to minimize variability due to external factors.

**Sample Collection and Handling**

Blood samples were collected by jugular venipuncture using Vacutainer tubes (BD Vacutainer®, Franklin Lakes, NJ, USA) with clotting activator and gel separator. Approximately 10 ml of blood was drawn from each horse and allowed to clot before centrifugation at 3500 rpm for 10 min to separate the serum. The serum was then stored at -20°C until analysis.

Saliva samples were collected using sterile cotton swabs. The swabs were placed in the horse's mouth for 60 seconds to absorb a sufficient amount of saliva, and then transferred to Salivate tubes. The saliva was immediately frozen at -20°C until further analysis.

All samples were packed with ice gel packs (-20°C) in insulated shipping containers (ThermoSafe®) and transported via express courier service (JNE Express) to the laboratory, maintaining the frozen chain throughout the 24-hour delivery period. Upon arrival, samples were stored at -80°C until analysis.

**HSP70 Measurement via ELISA**

The serum and saliva concentrations of HSP70 were measured by a commercial ELISA kit (Horse HSP70 ELISA Kit, Abbexa Ltd, Cambridge, UK) according to the manufacturer’s specifications. The ELISA was performed using a microplate reader (BioTek ELx808) at a wavelength of 450 nm. The intra-assay and inter-assay coefficients of variation (CV) were below 10% and 15%, respectively, demonstrating reliability of the results.

## Ethical Considerations

The study protocol was given ethical clearance by the University of Nusa Tenggara Barat's Ethics Committee for Animal Research, Faculty of Veterinary Medicine (Approval No: FVM-NTB/2024-001). All procedures complied with ethical treatment of animals and attempts to minimize discomfort during sample collection proved compliant.

## RESULT AND DISCUSSION

The study analyzed HSP70 concentrations in blood serum and saliva samples from 15 working horses on Sumbawa Island, where environmental temperatures ranged between 30-33°C. These working horses are routinely exposed to high temperatures and high physical exertion, making them particularly susceptible to heat stress. Given the daily workload of these horses, their physiological response to heat stress is critical to understanding how they adapt to extreme conditions and how their welfare can be optimized. Additionally, variations in HSP70 levels could provide an objective basis for assessing animal welfare in tropical field conditions. HSP70 levels in both serum and saliva are summarized in Table 1.

**Tabel 1.** HSP70 levels in blood serum and saliva of working horses (ng/mL)

| Sample | HSP70 Level in Blood Serum (ng/mL) | HSP70 Level in Saliva (ng/mL) |
|---|---|---|
| 1 | 4.62 | 24.30 |
| 2 | 8.01 | 23.83 |
| 3 | 10.87 | 30.69 |
| 4 | 8.06 | 31.54 |
| 5 | 9.65 | 38.70 |
| 6 | 13.28 | 38.87 |
| 7 | 63.87 | 39.52 |
| 8 | 93.96 | 29.10 |
| 9 | 42.54 | 70.82 |
| 10 | 13.65 | 103.36 |
| 11 | 68.52 | 88.13 |
| 12 | 70.00 | 75.91 |
| 13 | 82.31 | 74.89 |
| 14 | 82.60 | 51.04 |
| 15 | 83.72 | 60.39 |

According to the data, there is a substantial discrepancy in the concentrations of HSP70 among individual horses. Serum HSP70 mean concentrations were 43.71 ng/mL ranging from Horse 1 at 4.62 ng/mL to Horse 8 with a concentration of 93.96 ng/mL. These extremes in variability of serum HSP70 implies certain horses were under excessive physiological stress than others as the variations between workload intensity, time exposed to high ambient temperature and physiological thermal resistance varies among horses, which could explain HSP70 variability.

HSP70s in serum have been reported to be influenced via prolonged exposure to environmental stressors like heat and workload (Ebisuda et al., 2023). Likewise, cattle have been reported to demonstrate an increase in serum HSP70s when caused by prolonged thermal exposure demonstrated a protection from protein denaturing and oxidative destruction (Olvera-Maneu et al., 2023). The serum HSP70 levels observed in Horses 7, 8, and 11-15 indicated these horses were possibly under increased

thermal and/or physical stress which required an associated cellular defense mechanism activated to maintain homeostasis.

Interestingly, a few horses showed low serum HSP70 levels, with Horse 1 at 4.62 ng/mL and Horse 2 at 8.01 ng/mL. This may indicate that these horses were less affected by heat stress due to less intense activity, higher thermal endurance, or more effective coping mechanisms. A study in dairy cows indicated that individuals with lower levels of HSP70 expression had greater heat tolerance. This may be because these cows have more developed physiological adaptations, such as more efficient sweating and respiratory cooling mechanisms (Lamy et al., 2017).

On the other hand, salivary HSP70 levels did not fluctuate nearly as much with average levels of 52.07 ng/ml. Significant salivary HSP70 values were found for Horse 10 (103.36 ng/ml), Horse 11 (88.13 ng/ml) and Horse 9 (70.82 ng/ml), suggesting that salivary HSP70 is a responsive biomarker to acute rather than chronic stress (Bazzano et al., 2024).

These findings extend and align with those of non-invasive biomarkers; salivary HSP70 indicates acute physiological stress, and serum HSP70 reflects chronic stress (Hu et al., 2020). As salivary secretion is subject to the influence of the autonomic nervous system, this would indicate that the salivary HSP70 concentration can increase quite rapidly in response to an acute stressor, such as heavy exercise or a sudden change in temperature.

According to the data shown in Table 1, monitoring HSP70 levels in both serum and saliva was valuable, as both biomarkers provided some similar, yet complementary information in evaluating acute to chronic stress in working horses. The stark differences in HSP70 concentrations across certain individual horses demonstrates the importance of developing individualized stress management strategies for raising horses, particularly when they are exposed to high workloads and extreme environments.

**Tabel 2.** Summary of HSP70 levels in serum and saliva (ng/mL)

| Sample Source | N | Sum | Mean |
|---|---|---|---|
| Serum | 15 | 655.63 | 43.71±34.83 |
| Saliva | 15 | 781.07 | 52.07±25.25 |
| Total | 30 | 1436.70 | 47.89±30.19 |

Table 2 shows that HSP70 concentrations in saliva were, on average, higher (52.07 ng/mL) than those found in serum (43.71 ng/mL), which indicates that saliva might be reflecting a more acute and sensitive response to stress, while serum might reflect a more cumulative burden of stress. In addition, the lower standard deviation of saliva (SD = 25.25) compared to serum (SD = 34.83) suggests that salivary HSP70 concentrations are less variable and more stable among individual horses, making saliva a better means of assessing stress in real time (Bazzano et al., 2024).

The high overall variability in serum HSP70 suggests that individual horses experience different levels of chronic stress, likely related to each individual horse's workload, metabolic efficiency, and ability to cope with heat stress. Notably, some horses had consistently higher serum HSP70 concentrations (i.e., Horse 8: 93.96 ng/mL; Horse 15: 83.72 ng/mL), indicating that these horses were likely subjected to significant physiological stress as a result of prolonged exposure to high ambient temperatures (30-33°C) and heavy workloads. For example, previous studies have shown that serum HSP70 concentrations were significantly increased when cattle were exposed to prolonged heat stress, suggesting that serum HSP70 may be a useful and

consistent biomarker of chronic heat stress exposure in working horses (Ebisuda et al., 2023).

When comparing both types of samples, the mean HSP70 concentration for the total means (serum + saliva) was 47.89 ng/mL with an overall standard deviation of 30.19, indicating significant variation in stress responses between individual horses. The large variation also suggests that some horses were under much greater stress than others due to differences in work intensity, genetics related to heat tolerance, and overall health status. Variability amongst equines has been observed in past studies, where the mechanism of individual variability was due to the latitude of thermoregulation and metabolic adaptation on the stress biomarker expression.

The lower variability in HSP70 concentrations in saliva suggests saliva may be a more reliable, consistent, and acute representative of stress than serum, where concentration levels can vary based on long-term adaptation and cumulative stress exposure. These findings are consistent with studies conducted in dairy cows, in which salivary biomarkers were more responsive to acute periods of stress and serum biomarkers were more indicative of prolonged periods of adaptation (Olvera-Maneu et al., 2023).

Our interpretation lends weight to the potential use of salivary HSP70 as an applied measure of stress monitoring; however, more specific and sensitive research to confirm its viability in equine species needs to be carried out. Given the salvaging HSP70 measurement process is non-invasive, it could be a way for horse owners to monitor stress over time. For example, a handler might monitor the levels of salivary HSP70 to determine if an individual or grouping of horses were experiencing acute stress to allow the handler to intervene by making changes like workload, offering drinks, or employing cooling approaches. Whereas serum HSP70 may indicate more sustained or cumulative load on horses, and may be used in assessing chronic exposure.

Table 2 indicates that there may benefits to combining saliva and serum analyses for monitoring stress in horses. Saliva may capture instantaneous stress responses, while serum may document chronic stress response or recovery. Combining the two forms of analysis may provide mutually informative data to managers for the welfare of working horses, especially those subject to heat stress. However, these conclusions should be validated further. the data indicates that HSP70 can be a potentially informative biomarker for stress in working horses experiencing elevated temperatures and workload. The differences in HSP70 levels in both biological fluids highlights the need to consider sample type within the context of stress evaluation. While saliva can provide immediate information about acute responses, serum may give an indication of physiological adaptation in response to prolonged exposure. This gives credence to the notion that the evaluation of biomarkers requires deeper insights.

This study confirms that HSP70 is a useful biomarker for evaluating stress in working horses, particularly in high temperature and high work load situations. The significant differences observed in HSP70 concentrations in serum and saliva reinforce the need to consider the chosen biological medium for assessing stress in working horses. While saliva can provide a more immediate response to acute stress, serum can provide insight into more chronic stressors that may have occurred over time. Therefore, both media can be equally useful and informative when monitoring the resulting physiological stress response in working horses.

Heat stress is a critical factor influencing HSP70 expression in animals, as evidenced by studies in cattle documenting increases in HSP70 in multiple biological fluids as environmental temperatures rise (Lamy et al., 2017). Similarly in horses,

ambient temperatures that are considered high (30-33°C) increase HSP70 production, likely as a cellular defense mechanism (Liu et al., 2024). Goats and cattle studies have shown a positive correlation between HSP70 expression and thermal stress, highlighting the important role of HSP70 as a molecular chaperone to prevent protein denaturation and oxidative stress associated with heat stress (Nagayach et al., 2017).

In addition to environmental temperature, workload intensity contributes to variability of HSP70 since horses that were exposed to a more physically intense task exhibited greater HSP70 expression. Dairy cattle and draft horses studies have shown work-induced stress significantly alters HSP70 expressing animals, demonstrating that exercising in heat creates physiological challenges for the horse and may combine their heat stress with work effort (Kang et al., 2023). Early observation of stress responses can be detected using non-invasive approaches, for example sample salivary HSP70 enabling timely workload and recovery decisions for the horse to improve animal welfare.

From the viewpoint of practicality, monitoring HSP70 levels from saliva provides a convenient and effective method for assessing stress on an ongoing basis. Saliva sampling is non-invasive and enables real-time information on acute stress responses; this is better than collecting serum which requires venipuncture and could provoke added stress through handling. Other studies with horses, cattle, and sheep have demonstrated that salivary biomarkers include similar reflexivity for physiological stress while limiting invasiveness (Ebisuda et al., 2023).

The applications of these findings are important for the management of horses raised in tropical climates that routinely experience heat stress. Management practices like workload adjustments, shaded rest areas, hydration practices, and electrolyte supplementation represent sound approaches to reduce the negative impacts of heat exposure over longer durations (Olvera-Maneu et al., 2023). Monitoring serum HSP70 levels over time can be used to identify horses that are at risk of long-term stress and require preventive interventions to maintain performance while ensuring animal welfare.

## CONCLUSION

This research emphasizes that HSP70 is an important biomarker for assessing stress in working horses, especially in hot climates. The results show that the saliva test works well for measuring immediate responses to stress, while serum remains useful for assessing long-term stress exposure. By regularly checking HSP70 levels, trainers can make better decisions about work schedules and care practices to optimize horses' well-being. The study also found that individual horses respond differently to stress, suggesting that management methods should be tailored to each animal. Future research should investigate why some horses deal with heat better than others and develop specific strategies to help working horses cope with stress. These findings are especially important for tropical areas like Sumbawa where horses work in difficult conditions.

## RECOMMENDATION

Further longitudinal research is recommended to monitor HSP70 levels in working horses during wet and dry seasons. The goal is to see more complete patterns of acute and chronic responses to heat stress, so that HSP70 can be used as a more accurate biomarker of stress in the management of equine welfare in tropical climates.

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