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The Association of Single Nucleotide Polymorphism -69T>G HSPA1A Gene with Bali Cattle Heat Tolerance

I. Suhendro[a,b], J. Jakaria[b], R. Priyanto[b], W. Manalu[c], & R. R. Noor[b,*]
[a]Postgraduate student of Animal Production and Technology, Faculty of Animal Science, IPB University
[b]Department of Animal Production and Technology, Faculty of Animal Science, IPB University
[c]Department of Anatomy, Physiology, and Pharmacology, School of Veterinary Medicine and Biomedical Sciences, IPB University
Jalan Agatis, Kampus IPB Dramaga Bogor 16680, West Java, Indonesia
*Corresponding author: ronny_noor@yahoo.com
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ABSTRACT

Heat shock protein plays an essential role in thermoregulatory during heat stress responses. This study aims to determine the association of single nucleotide polymorphism (SNP) -69T>G in the promoter region of the heat shock protein 70 member 1A (HSPA1A) gene on heat tolerance in Bali cattle. One hundred and sixteen heads of Bali cattle were collected from different locations such as Pangyangan, Bali Island; Serading, Sumbawa Island; and Sembalun, Lombok Island. The SNP was analyzed by genotyping using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), which used BstUI enzyme restriction. Physiological responses including respiration rate (Rr), rectal temperature (Tr), heart rate (Hr), heat tolerance coefficient (HTC), and blood glucose level (Glu) were measured. Association analysis was conducted using a general linear model by setting genotype, altitude, and sex as factors. The SNP -69T>G variant of HSPA1A gene found in this study were wild type (TT) with 144 bp & 498 bp; GG with 144, 236, & 262 bp; and TG with 144, 236, 262, & 498 bp. Bali cattle with the GG genotype had lower ($p<0.001$) Rr and HTC compared to the other genotypes. It could be concluded that physiological performances were lower at high altitudes, and the SNP -69T>G HSPA1A was associated with the physiological performances of Bali cattle. SNP -69T>G of HSPA1A could be utilized for candidate marker-assisted selection of Bali cattle to improve the performance of heat tolerance.

Keywords: Bali cattle; heat tolerance; HSPA1A gene; promoter region; SNP

INTRODUCTION

Heat stress causes a substantial economic loss to livestock producers by billions of dollars (Key *et al.*, 2014). This substantial economic loss is caused by heat stress that decreases feed intake, growth and development, milk production, and beef quality (St-Pierre *et al.*, 2003; Summer *et al.*, 2019). Cattle respond to heat stress in various ways, including behavioral, physiological, cellular, and molecular alterations (Archana, 2017). Microclimate environmental factors, such as temperature and humidity, influence thermotolerance ability through heat dissipation, panting, and sweating.

Heat shock protein (HSP) has a crucial role in cell survival (Ikwegbue *et al.*, 2018) since this protein is an essential biomarker of heat stress (Abdelnour *et al.*, 2019) to reduce the destructive effects of heat stress. HSP70s function as a housekeeping and quality controller due to their roles in signal transduction pathways, protein structure correction, and repairing misfolded proteins in response to stressors, especially heat stress (Rosenzweig *et al.*, 2019).

The diversity of the HSP70 gene is widely used for association studies of thermotolerance traits in cattle (Basiricò *et al.*, 2011; Bhat *et al.*, 2016; Dikmen *et al.*, 2015). Mutations in the 5 UTR region were associated with blood biochemical parameters and thermoregulatory ability of lactating Holstein cattle in China (Abbas *et al.*, 2020). HSP70 is classified into subfamilies based on minor structural changes in its nucleotide bases, including HSPA1A, HSPA1B, and HSPA1L. The HSPA1A gene subfamily was also associated with cattle performance (Cochran *et al.*, 2013; Kerekoppa *et al.*, 2015; Öner *et al.*, 2017). Suhendro *et al.* (2021) found a base mutation of thymine to guanine in the HSPA1A gene at 5′ untranslated region (5′UTR) of transcription position -69 (g.-69T>G).

Numerous genes utilize the 5′UTR region for transcriptional regulation since there is an abundance of binding sites for diverse transcription factors (Barrett *et al.*, 2012; Hinnebusch *et al.*, 2016). The substitution mutation of g.-69T>G coincided with the binding site for the CAAT box and the NF-Y box (Figure 1). The determination of the transcription factor is based on their consensus motif. The consensus motif for CAAT box is

"RRCCAATSR" (reverse complement = YSATTGGYY) (Bucher, 1990), and it is placed between -212 to -57 nucleotides relative to the transcription initiation site (Bucher, 1990). NF-Y exhibits a high affinity to the consensus motif "RRCCAATSRGMR" (reverse complement = YKCYWATTGGYY) (Matuoka & Chen, 2002).

Although no previous study has associated these SNPs with cattle performance, this SNP with CCAAT box – NF-Y is hypothesized to play a role in responding to heat stress via HSPs (Li *et al.,* 2018). NF-Y box is important to cell responses since it is involved in cell proliferation and differentiation in various diseases, stress response, growth, and development (Imbriano *et al.*, 2001; Yamanaka *et al.,* 2008).

The genetic diversity of the HSPA1A gene in the Bali cattle population, especially SNP g.-69T>G, and its relationship to heat stress are still unknown. Therefore, this study aimed to investigate the physiological responses during heat stress and its association with the single nucleotide polymorphism g.-69T>G HSPA1A promoter gene in Bali cattle.

MATERIALS AND METHODS

Animal and Physiological Responses Measurements

The study was conducted from June to December 2021. The experiment was conducted in compliance with the Animal Ethics Committees at Udayana University in Denpasar, Indonesia (Code ID: B/184/un14.2.9/pt.01.04/2021). A total of 108 healthy matured Bali cattle (male and female) were used, consisting of 37 Bali cattle sampled in Pangyangan, Bali Island (8°25'3" S, 114°51'49" E, and 46 m altitude), 37 Bali cattle sampled in *Serading* Sumbawa Island (8°34'04" S, 117°29'48" E, and 50 m altitude), and 34 Bali cattle sampled in *Sembalun*, Lombok Island (8°21'48" S, 116°31'49" E, and 1.186 m altitude). Microclimate measurements were carried out at each location using a Thermo-Hygrometer. Furthermore, the relative humidity (Rh) and ambient temperature (Ta) data were used to calculate the temperature-humidity index (THI) in each location. The highland of Sembalun has a lower THI (70.51) than other altitudes in Serading (78.79) and Pangyangan (77.03) (Table 2). All experimental cattle in each location were housed in a semi-intensive, and they were fed ad libitum using natural feed made from agricultural products and agricultural waste. Furthermore, physiological responses of respiratory rate (Rr), heart rate (Hr), rectal temperature (Tr), and blood glucose (Glu) concentrations were measured during the sampling months in October. These parameters were measured for about five minutes after the animals were placed in the squeeze chute. The heat adaptability of experimental Bali cattle was calculated using the coefficient of heat tolerance (HTC) known as the Iberian heat tolerance: HTC= Tr/38.3 + Rr/23, where Tr= rectal temperature; Rr= respiration rate/minute; 38.33= Normal Tr (°C); 23= normal Rr/min (Singh *et al.,* 2015). Animals with an HTC value equal to 2 show tolerant adaptability, and animals with value larger than 2 indicate lower adaptability (Singh *et al.,* 2015).

Procedure of Genomic DNA Extraction and PCR Amplification

Total DNA was extracted from whole blood samples using the Geneaid® DNA extraction kit procedure (Geneaid Biotech Ltd., Taiwan). The primer was manually designed based on GenBank® of access number AY149618.1. for the targeted region (5' flanking and promoter of HSPA1A) using Primer3, which was further evaluated using Primer Stat. The product length from a forward primer (5'-GTTTGATACG GTTCGGATGG-'3) and reverse primer (5'- GAAGCTTATC TCGGAGCC-'3) was 642 bp. DNA amplification was done using the polymerase chain reaction (PCR) method with a Master Cycler Gradient machine (ESCO, Singapore). Each reaction was in a final volume of 15 µL, having 1 µL of sample DNA, 6.1 µL of nuclease-free water (NFW), 0.2 µL of forward primer, 0.2 µL of reverse primer, and 7.5 µL of GoTaq® Green Master Mix (Promega, USA). The reaction conditions were 95 °C for 5 min; 94 °C for 10 s; 55 °C for 20 s; 72 °C for 30 s; the number of the cycle was 30 cycles; 72 °C for 5 min. The amplification product was then visualized on 1.5% agarose gel, stained using DNA FlouroSafe staining, and then photographed using a UV Transilluminator (Biorad™, California, USA).

Procedure of PCR-RFLP and Genotyping

Genotyping was performed using the polymerase chain reaction restriction fragment length polymorphism (PCR RFLP) procedure with restriction endonuclease enzyme of BstUI (CG/CG), designed manually using NEBcutter V2.0 (Vincze *et al.,* 2003). Each reaction had 5 µL of the amplicon, 0.5 µL of enzyme restriction, 0.6 µL of buffer enzyme, and 0.9 µL of NFW, which were incubated at 37 °C for four hours. Visualized products were performed by electrophoresis using 2% gel agarose. The identification of genotypes was figured out based on the pattern of emerging bands.

Statistical Analysis

The genotype and allele frequencies SNP of g.-69T>G HSPA1A gene was calculated using the formula of Allendorf *et al.* (2013). Allele frequencies were $X_i = \frac{(2n_{ii}+\sum_{i\neq j} n_{ij})}{2N}$ and genotype frequencies was $X_{ii}= n_{ii}/N$, where n_{ii} was the number of individuals with ii genotype; n_{ij} was the number of individuals with ij genotype; and N was the population size. Hardy Weinberg Equilibrium was calculated in a model of $x^2 = \sum_{k=0}^{n} \frac{(O-E)^2}{E}$, where O was total of observations; E was total of expectations.

The experimental design used a completely randomized design (CRD) one way ANOVA, with HSPA1A genotypes variants on physiologies performances representing as the independent variable. The data analysis was conducted using R Studio® General Linear Model (R Studio, BC, Boston, MA, 2020), and the mean values were compared with the LSD *post hoc* range test. The statistical model for physiological responses in different

altitudes is $Y_{ijk} = \mu + a_i + s_k + \epsilon_{ijk}$. The statistical model for the association of HSPA1A genotype with phenotypic responses was $Y_{ijk} = \mu + g_i + s_k + \epsilon_{ijk}$. Where Y_{ijk} *was* physiological responses observed traits, μ was population mean, a_i was altitude effect, s_k was sex effect, g_j was genotype effect, and ϵ_{ijk} was error.

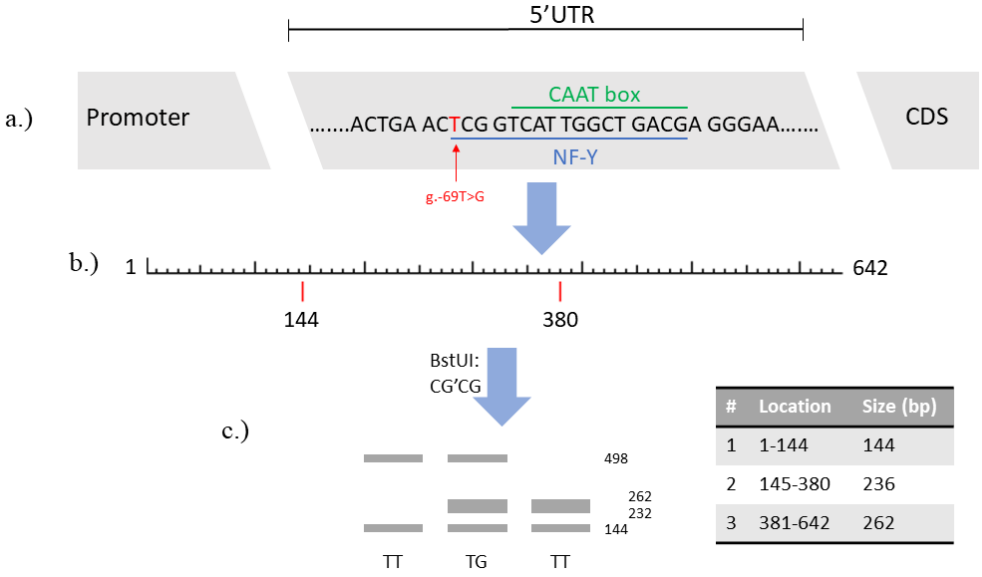
RESULTS

The genotyping of the HSPA1A gene in g.-69T>G with BstUI restriction enzyme produced three genotypes, namely wild type (TT: 144 & 498 bp), homozygous (GG: 144, 236, & 262 bp), and heterozygous (TG: 144, 236, 262, & 498 bp) (Figure 1 and Figure 2). This genotype variation was obtained from the difference in the CG'CG cutting site of the target gene. There are two CG'CG cut sites at positions 144 bp and 380 bp (Figure 2), although the 144 bp cut can be ignored as it is not a genotyping target.

The HSPA1A SNP in g.-69T>G was polymorphic which the genotype frequencies were 45.4%, 46.3%, and 8.3% for TT, TG, and GG, respectively (Table 1). The frequency of T allele (0.69) was higher than that of G allele (0.31). All these genotype frequencies were in Hardy Weinberg equilibrium. Bali cattle raised in the lowland of Pangyangan and Serading have slightly excess heterozygotes (51% and 54%, respectively), while in Sembalun, have heterozygosity deficit (Ho= 32%). This heterozygous advantage is probably related to the adaptability of cattle since the heterozygous allele brought the natural selection advantages for an animal in diverse environments (Allendorf *et al.*, 2013).

The factors of altitude (Table 2) and genetics (Table 3) significantly affected the physiological responses in Bali cattle, but sex did not affect these responses (data not shown). This altitudinal factor clustered Bali cattle into hugely different ($p<0.001$). Sembalun was classified as a highland since that area had a much lower THI; meanwhile, Pangyangan and Serading have about the same physiological responses; hence they were classified as lowland locations.

The significant association of SNP-69T>G HSPA1A with physiological responses of Bali cattle in various altitudes was presented in Table 3. The genotype variant had a significant ($p<0.001$) effect on physiological responses. In general, the LSD *post hoc* showed that



#	Location	Size (bp)
1	1-144	144
2	145-380	236
3	381-642	262

Figure 1. The gene structure of HSPA1A and the genotyping strategy. a) SNP position in HSPA1A gene structure and transcription factor attachment sites for CAAT box and NF-Y box, b) sequence of HSPA1A gene target which has a CGCG base, c) results of BstUI restriction enzyme and visualization of genotyping.

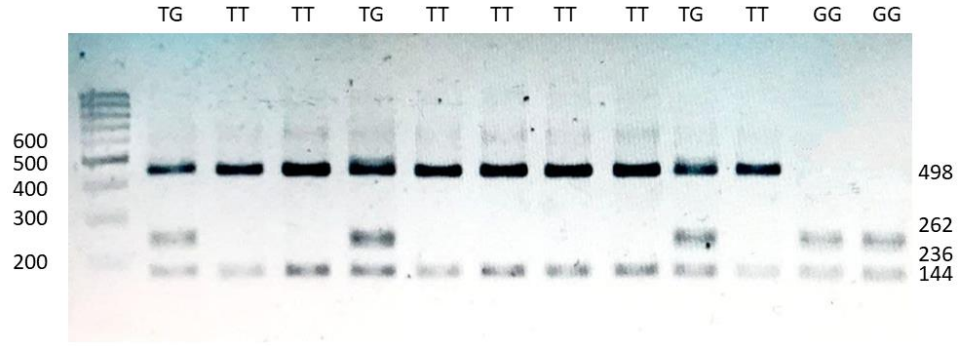


Figure 2. The visualization of HSPA1A fragmentation by BstUI restriction enzyme in the Bali cattle population (M: ladder of 100 bp; TT, TG, GG = genotype).

Table 1. Allele and genotype frequency of g.-69T>G HSPA1A gene of Bali cattle under different altitude

Area (no. samples)	Allele freq.		Genotype freq. (no. samples)			Heterozygosity		Chi-square
	T	G	TT	TG	GG	Obj.	Exp.	Prob.
Pangyangan (37)	0.69	0.31	0.43 (16)	0.51 (19)	0.05 (2)	0.51	0.43	ns (0.26)
Serading (37)	0.70	0.30	0.43 (16)	0.54 (20)	0.03 (1)	0.54	0.42	ns (0.09)
Sembalun (34)	0.66	0.34	0.50 (17)	0.32 (11)	0.18 (6)	0.32	0.45	ns (0.09)
Combined (108)	0.69	0.31	0.454	0.463	0.083	0.46	0.43	ns (0.47)

Note: The Chi-square probability value was ns (non-significant) when greater than 0.05.

Table 2. Physiological responses of Bali cattle at different altitudes

Physiological responses	Location (THI value)			Mean	*P*-value
	Pangyangan (77.03±2.99[a])	Serading (78.79±3.67[a])	Sembalun (70.51±4.31[c])		
Resp. rate (beats/min)	41.25±80[a]	37.08±8.77[b]	24.63±3.46[c]	34.62±9.35	<0.001
Heart rate (beats/min)	72.80±9.85[a]	66.54±18.5[a]	60.92±12.81[b]	67.18±13.9	0.0061
Rectal temperature (°C)	38.78±0.44[a]	38.38±1.89[a]	37.90±0.56[b]	38.47±0.82	<0.001
Heat tolerance coefficient (HTC)	2.81±0.35[a]	2.59±0.49[b]	2.07±0.16[c]	2.51±0.42	<0.001
Blood glucose (mg/dL)	48.55±8.29[a]	47.52±8.14[a]	39.04±12.85[b]	45.21±8.62	<0.001

Note: Means in the same row with different superscripts differ significantly (p<0.05).

Table 3. The association of HSPA1A gene polymorphism with physiological responses of Bali cattle

Physiological responses	The genotype of -69T>G (no. samples)			*P*-value
	TT (49)	TG (50)	GG (9)	
Resp. rate (beats/min)	33.96±10.20 [ab]	36.16±8.45 [b]	29.00±7.33 [a]	0.009
Heart rate (beats/min)	66.94±13.34	68.64±14.33	59.50±13.77	0.184
Rectal temperature (°C)	38.37±0.86	38.54±0.80	38.68±0.66	0.313
Heat tolerance coefficient (HTC)	2.48±0.46 [ab]	2.58±0.38 [b]	2.27±0.33 [a]	0.009
Blood glucose (mg/dL)	45.52±8.34	45.8±7.93	39.88±12.99	0.150

Note: Means in the same row with different superscripts differ significantly (p<0.05).

Bali cattle with GG genotype had a lower respiration rate and heat tolerance coefficient when compared to the other genotypes. These results indicated that the HSPA1A gene might play an important role in heat tolerance.

DISCUSSION

The physiological response of Bali cattle in this study was significantly different (p<0.0001) according to altitude, wherein cattle raised in the lowland were found to be suffering from acute heat stress (Table 2). This heat stress was reflected by the average Rr and HTC values that exceed the normal threshold. On the other hand, Bali cattle raised in comfortable conditions in the highlands could maintain their normal physiological responses. The normal physiological for Rr of cattle reared in semi-intensive should be around 24-36 times/minutes (Jackson & Cockcroft, 2007), and HTC is about 2 (Singh *et al.*, 2015).

The other physiological traits (Hr, Tr, and Glu) were normal in both locations. Usually, cattle not subjected to heat stress have heart rates of 60-80, Tr below 39.00 (Jackson & Cockcroft, 2007), and blood glucose levels of 40 to 60 mg/dL (Mair *et al.*, 2016). However, Bali cattle in the highlands showed a significant variation, with a lower response than the normal threshold. The Hr in Pangyangan and Serading were quite normal with 72.80±9.85, 66.54±18.5, respectively. However, Sembalun showed a slightly below normal (60.92±12.81). The blood glucose level of Bali cattle reared in Sembalun showed a similar phenomenon.

The animals possess physiological responses to maintain heat balance and homeostasis in changing climatic conditions (Das *et al.*, 2016). This ability of animals to cope with extreme heat stress demonstrates that these animals are more energy-efficient and capable of surviving in a changing climate (Lees *et al.*, 2019). Since animals with a low physiological response require less energy to maintain their body conditions (Li *et al.*, 2020), the excess energy can be allocated for production purposes. Therefore, livestock was better housed at higher altitudes with a more comfortable THI, where the animal spent less energy for metabolism in this area.

The polymorphism of SNP g.-69T>G HSPA1A in this study produced three genotypes, namely TT, TG, and GG. This transversion mutation had a significant association with respiration rate and heat tolerance coefficient traits, where the GG genotype had lower (p<0.05) values than those of the TT and TG (Table 3). These physiological traits were often used as biomarkers for primary or secondary indicators of heat stress

(Gourdine *et al.,* 2017; Carabaño *et al.,* 2019) and disease (Tulaimat *et al.,* 2014). Rr and Tr have been reported as biomarkers for assessing the impact of heat stress on animals (Shilja *et al.,* 2016). Rr is commonly used as a physiological marker to evaluate the heat stress response in livestock (Dalcin *et al.,* 2016), while under stress conditions, ruminants adapt by enhancing the evaporative cooling mechanism by increasing the Rr to reduce the excess heat load (Singh *et al.,* 2016). Heat stress causes the Tr to dissipate heat loads (El-Tarabany *et al.,* 2017). Increased Tr implies that an animal cannot maintain a normal body temperature under heat stress (Wang *et al.,* 2020). Rr and Tr are related to HTC; the higher Rr and Tr could lead to heat stress because the cattle have a higher HTC value. HTC measurements in this study were above a normal HTC index (HTC ≥ 2), according to Singh *et al.* (2015). The HTC value should generally be lower than 2 for thermotolerant cattle. However, raising livestock in the tropics is much more difficult due to the challenging environment. Nevertheless, the HTC values of Bali cattle in this study were still lower than Madura cattle (2.62±0.2) (Yosit *et al.,* 2016) and FH cattle (2.9±0.48) (Mariana *et al.,* 2020), which were raised in the same tropical climate.

The polymorphism studies of the HSP70 gene in mammals were mostly conducted in promoter and upstream regions. Mutations in this region were found to be associated with thermotolerance (Basiricò *et al.,* 2011; Xiong *et al.,* 2013; Hu *et al.,* 2019), blood biochemical (Abbas *et al.,* 2020), reproductive traits (Rosenkrans *et al.,* 2010; Said & Putra, 2018), and milk composition (Brown *et al.,* 2010; Mariana *et al.,* 2020). Although mutations in the upstream open reading frames (uORFs) did not change amino acids or proteins, they might influence mammal phenotype and disease susceptibility through the possibly reduced protein expression by up to 30%-80% (Calvo *et al.,* 2009).

The g.-69T>G HSPA1A (dbSNP = rs797598758) was a novelty in cattle studies since there was no evidence of any association with cattle phenotype. Mutations in this site became crucial since it coincidentally binds to transcription factor NF-Y (Suhendro *et al.,* 2021). NF-Y is a crucial transcription factor involved in the proliferation and differentiation of cells in various diseases, stress responses, growth, and development (Li *et al.,* 2018). NF-Y is found in various organisms attached to the cis-acting CCAAT box in the promoter region of the HSPs gene.

There were several other studies discussing mutations in the HSP70 gene that binds to the other transcription factors, such as the AP box transcription factor (g.895C>-), which was found to be associated with heat stress on peripheral blood mononuclear cells (PBMC) (Basiricò *et al.,* 2011), thermal stress response traits (Deb *et al.,* 2013), and milk production traits (Deb *et al.,* 2013; Mariana *et al.,* 2020).

Bali cattle with the GG genotype exhibited better thermotolerant traits due to their reduced RR and HTC values when compared to Bali cattle with the normal TT genotype and heterozygous TG. This alteration in physiological response might be well due to mutations that disrupt the binding site in the CCAAT box and NF-Y, allowing cattle to become more tolerant, then could be related to the expression of the HSP70 gene. The increased HSP70 gene expression indicates that cattle can better tolerate heat stress (Parmar *et al.,* 2015). In comparison, Yamanaka *et al.* (2008) demonstrated that the decreased NF-Y binding resulted in the decreased HSP70 expression. However, it is unknown whether this mutation decreases or increases HSP70 expression.

CONCLUSION

Bali cattle raised at higher altitudes had a significantly lower physiological response. The SNP g.-69T>G of the HSPA1A gene was polymorphic in Bali cattle raised in different altitudes. The variant genotypes of the HSPA1A gene had a significant association with physiological responses, such as respiration rate and heat tolerance coefficient. According to the findings, the HSPA1A SNP g.-69T>G with the GG genotype could be considered as a candidate marker for selecting Bali cattle with low physiological responses.

CONFLICT OF INTEREST

Jakaria and Wasmen Manalu serve as editors of the Tropical Animal Science Journal, but have no role in the decision to publish this article. The authors also declare that there is no conflict of interest.

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