Differences in Physiological Characteristics and Heat Shock Protein Expression in Taiwan Swamp Buffaloes During Winter and Summer Seasons

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Abstract: *Background*: This study examined the respiratory rate, rectal temperature, and expression levels of heat shock protein 70 (*HSP70*) and heat shock protein 90 (*HSP90*), as revealed by ELISA, in Taiwan swamp buffaloes (*Bubalus bubalis*, swamp-type) during the winter (February) and summer (August) seasons of 2022 in Taiwan.

Methods: Data were collected from Taiwan swamp buffaloes during the winter and summer seasons. Respiratory rate, rectal temperature, and protein expression levels were measured and analyzed.

Results: The results revealed age-related differences in response to changes in environmental temperature. In winter, buffaloes aged <1 year exhibited significantly higher respiratory rates, rectal temperatures, and heat tolerance coefficients than female buffaloes aged 14 to 20 years ($P < 0.05$). In the summer season, buffaloes aged <1 year had significantly higher rectal temperatures ($P < 0.05$) and higher expression levels of *HSP70* (from ELISA) than female buffaloes aged 6 to 9 years and 14 to 20 years ($P < 0.05$).

Conclusion: The findings suggest that the age of Taiwan swamp buffaloes affects their physiological responses to heat stress, with younger buffaloes exhibiting greater physiological reactions to heat stress than older buffaloes.

Keywords: Taiwan swamp buffalo, age, respiratory rate, rectal temperature, heat shock protein, ELISA.

INTRODUCTION

Under exposure to heat stress, cattle typically exhibit decreased dry matter intake (DMI), growth, reproductive performance, feed conversion efficiency, and reductions in the quantity and quality of dairy products [1]. Common physiological responses include increased respiratory rate, elevated rectal temperature, changes in posture, and behaviors such as seeking shade and water sources. Eigenberg *et al.* [2] investigated the effect of shading devices on cattle's respiratory rates, and the results revealed that in summer, the average per-minute daytime respiratory rate of cattle without shade was 16 breaths higher than that of cattle provided with shade.

Different coat colors affect the degree of heat stress response in cattle. Brown-Brandl *et al.* [3] compared the heat stress responses of cattle with four coat colors in summer. Angus cattle (black coat) exhibited higher respiratory rates, panting severity, and surface temperatures than cattle with dark red, light brown, and white coats. Heat stress not only increased water intake and standing behavior but also reduced feed intake and lying behavior, indicating that cattle with darker coats are more affected by changes in the environmental temperature than those with lighter coats. Due to their black-gray skin and sparse hair, water buffaloes absorb a significant amount of heat [4].

Additionally, the sweat gland density of water buffaloes is only one-sixth of that of dairy cattle [5], resulting in lower heat dissipation capabilities for water buffaloes than for other cattle breeds. If appropriate cooling is not achieved for water buffaloes under high temperatures, a range of negative effects can occur, including decreased feed intake; disrupted protein, water, energy, and mineral balances; and altered hormone secretion and blood circulation, leading to impaired growth and reproductive capabilities [6]. Consequently, during hot weather, water buffaloes often seek shade under shelters or trees or immerse themselves in water pools to decrease their body temperature.

In addition to the observable external responses to heat stress, biomarkers in the blood can serve as indicators of the stress levels experienced by animals [1]. Heat shock protein 70 (*HSP70*) is a characteristic cellular marker in water buffaloes [7]. As a molecular chaperone, *HSP70* plays a crucial role in cellular heat tolerance and regulates apoptosis, immune function, and heat stress response. Variations in its gene's flanking and promoter regions have been linked to several traits such as heat tolerance, weaning weight, milk yield, fertility, and disease resistance in various

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livestock species [8]. Heat shock protein 90 (*HSP90*) regulates several cellular processes, including cell cycle control, signal transduction, stress response, and the management of protein folding, degradation, and transport. These essential roles highlight HSP90 as a crucial molecular chaperone for maintaining cellular stability under stress conditions [9]. Liu *et al.* [9] found that during summer in China (with a temperature-humidity index (THI) value of 82.95 ± 0.77), crossbred water buffaloes (Nili-Ravi × Murrah) exhibited significantly lower rectal temperatures, surface temperatures, and respiratory rates than Mediterranean buffaloes. Moreover, as revealed by ELISA, the expression levels of *HSP70*, *HSP90*, and cortisol were significantly higher in crossbred water buffaloes, indicating species differences in the expression of *HSP70* and *HSP90* under heat stress.

Kumar *et al.* [10] studied 100 Sahiwal cows; they analyzed single nucleotide polymorphisms (SNPs) in the target regions of the *HSP90AA1* gene (exons 3, 7, 8, and 11) and examined their association with heat tolerance traits in Sahiwal cows. Respiratory rates and rectal temperatures were recorded in the winter, spring, and summer seasons, and the heat tolerance coefficient (HTC) was also calculated. The results revealed five SNPs, namely A1209G, A3292C, T4935C, T5218C, and A5224C, in the *HSP90AA1* gene target regions. Only the AA genotype of A1209G and the AC genotype of A3292C were significantly associated with heat tolerance, indicating that Sahiwal cows with the AA and AC genotypes have better heat tolerance; thus, these genotypes are beneficial for improving heat tolerance traits in Sahiwal cattle. Chuang *et al.* [11] analyzed the presence of single nucleotide variants (SNVs) in *HSP70* and *HSP90* at specific loci (*HSP70* g.24927355 T>C, *HSP70* g.24927424 G>A, *HSP70 E2* g.24928813 C>T, *HSP90* g.17755706 G>A, and *HSP90* g.17756478 T>C) in swamp-type water buffaloes. However, no significant differences were observed between different genotypes in terms of physiological values in the winter and summer seasons or in the plasma expression levels of *HSP70* or *HSP90* in the tested buffalo population.

This study aims to investigate how age influences the physiological responses of Taiwan swamp buffaloes (*Bubalus bubalis*) under environmental heat stress. Specifically, it examines the expression levels of heat shock proteins *HSP70* and *HSP90* in the plasma of buffaloes across different age groups during the winter and summer seasons, as measured by ELISA. These findings will serve as supplementary information for management practices, such as providing targeted cooling strategies for buffaloes of different ages during periods of environmental stress. Furthermore, the study explores the genetic polymorphisms of *HSP70* and *HSP90* in Taiwan swamp buffaloes, aiming to identify genetic markers associated with heat stress resistance. This information could contribute to the preservation of genetic resources and inform selective breeding programs to enhance stress tolerance, addressing the challenges posed by the increasing climate change trends in Taiwan.

MATERIALS AND METHODS

Experimental Animals and Design

Experimental buffaloes were divided into three groups: Group A consisted of eight buffaloes aged <1 year (four male and four female buffaloes), Group B comprised eight female buffaloes aged 6 to 9 years, and Group C included four female buffaloes aged 14 to 20 years, with a total of 20 buffaloes. During the experiment, the buffaloes were housed in an open barn, with shade canopies measuring 15 m in length and 8 m in width and an exercise area measuring 60 m in length and 40 m in width, and the barn was equipped with flowing water. Fresh concentrate feed and clean drinking water were provided daily, along with *ad libitum* access to hay. The housing areas remained the same during the study period.

The animal experiments were conducted at the Hualien site of the Eastern Region Branch of the Taiwan Livestock Research Institute, Ministry of Agriculture. The animal housing, usage, and experimental procedures were approved by the Institutional Animal Care and Use Committee of the Eastern Region Branch of the Taiwan Livestock Research Institute (HUAIACUC11104) and were conducted in accordance with their guidelines.

Measurement Items and Methods

Environmental Temperature and Humidity

A temperature and humidity data logger (EL-SIE-2, EasyLog, China) was installed in the buffalo barn to record the environmental temperature and humidity at 13:00 for 5 full consecutive days in February and August 2022. The temperature–humidity index (THI) was then calculated using the following formula [12,13]:

$$THI= (1.8 \times T + 32) – ((0.55-0.0055 \times RH) \times (1.8 \times T-26))$$

T: ambient temperature (˚C); RH: relative humidity (%)

Measurement of physiological values in winter and summer seasons:

In the winter season (February) and the summer season (August) of 2022, the experimental buffaloes were guided to the working area in the buffalo barn at 1300 h for 5 consecutive days. Once the buffaloes were restrained, their respiratory rate (measured by counting the flank movements in 1 minute while the buffalo was standing still) and rectal temperature were recorded. These values were then converted into the HTC. An HTC value of 2 indicates maximum heat tolerance, and HTC values exceeding 2 indicate lower heat tolerance. The HTC was calculated using the following formula [14,15]:

$$RT/38.33 + RR/23$$

RT: Rectal Temperature (˚C); RR: Respiration Rate/min.

DNA Extraction and Analysis of Single Nucleotide Variations

Three milliliters of venous blood were drawn from the jugular vein of the experimental buffaloes into EDTA tubes containing an anticoagulant (BD Vacutainer® Lithium Heparin). DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Germany). Primers from a previous study [11], which successfully amplified the *HSP70* and *HSP90* gene fragments (Table **1**), were used for polymerase chain reaction (PCR). The PCR products were then sequenced by Genomics BioSci & Tech Co., Ltd. To confirm the target gene fragments, the sequencing results were compared using the BLAST function on the NCBI website. The sequences were then analyzed using Chromas 2.6.6 software (Technelysium, Australia) to identify SNVs in the experimental buffaloes.

ELISA Expression Levels of HSP70 and HSP90

In the experimental period, 10mL of venous blood was drawn from the jugular vein of the experimental buffaloes into EDTA tubes containing an anticoagulant (BD Vacutainer® Lithium Heparin). The blood samples were centrifuged at 3,000 rpm for 15 min using a centrifuge (AN5-2VG-KUBOTA-5800, Kubota, Japan), and the plasma was collected. The expression levels of *HSP70* and *HSP90* in the plasma in the winter and summer seasons were measured by Lezen Reference Laboratories using the bovine *HSP70* ELISA kit (CUSABIO, Bovine Heat Shock Protein 70 ELISA kit, 96 tests, USA) and the bovine *HSP90* ELISA kit (CUSABIO, Bovine Heat Shock Protein 90-alpha [*HSP90AA1*] ELISA kit, 96 tests, USA), respectively.

Statistical Analysis

The data obtained from the experiment were analyzed using the statistical software IBM SPSS Statistics version 22 [16]. Independent samples *t*-tests were used to examine the differences between the data obtained during the winter and summer seasons. One-way analysis of variance and the LSD test method was

Table 1: Primers for Amplifying and Sequencing *HSP70* and *HSP90* from Taiwan Swamp Buffaloes

Primer name	Primer Sequence (5'→3')	Size (bp)	References/GenBank accession No.
HSP70-F1	AAACAGCAGCCTGGAGAGAG	942	chromosome 2 HSP70 Exon 1 (NC_037546.1) [11]
HSP70-R1	TGGCTGATGTCCTTCTTGTG		
HSP70-F2	GGACTTGGGTCTTGCCCTAT	302	chromosome 2 HSP70 Exon 2 (NC_037546.1) [11]
HSP70-R2	CGCATTTATCATTTTCTTTTTATTCC		
HSP90-F1	TCACCCAGGAGGAATATGGAG	692	chromosome 23 HSP90 Exon 7 (NW_001494158.3) [34]
HSP90-R1	AGAAGGACCGATTTTCTCACC		
HSP90-F2	TCGGTCCTTCTGTTGAATCC	669	chromosome 23 HSP90 Exon 8 (NW_001494158.3) [11]
HSP90-R2	CCTGCTCTTTGCTCTCACCT		

used to determine whether significant differences were present in the mean data values of the experimental buffaloes across different age groups in the winter and summer seasons, with significance set at $P < 0.05$.

RESULTS AND DISCUSSION

Effects of Environmental Changes in Winter and Summer Seasons on Rectal Temperature, Surface Temperature, and Respiratory Rate in Taiwan Swamp Buffaloes Across Different Age Groups

According to the 2022 temperature and humidity records from Taiwan's Central Weather Administration (https://www.cwa.gov.tw/V8/C/), the average temperature in Hualien in January and February was 18.65 ± 0.07 °C, marking this period as the coolest of the year, with an average THI of 64.80 ± 0.03. From July to August, the average temperature was 29.20 ± 0.01 °C, and the average THI was 81.49 ± 0.41. In July and August 2022, the THI values exceeded 80, indicating that this period was the hottest of the year (Figure **1**). This study defined February and August as the winter and summer seasons, respectively. Environmental temperature, humidity, and THI values were collected at 1:00 PM for 5 consecutive days in the winter season (February) and the summer season (August) in 2022. The average environmental temperature in the winter season was 20.05 ± 3.18 °C, with an average THI of 66.41 ± 4.60. In the summer season, the average environmental temperature was 28.70 ± 0.30 °C, with an average THI of 79.38 ± 1.52 (Table **2**). The THI can be used as an indicator to assess the impact of climatic conditions on heat stress in animals [17]. A THI of ≤74 indicates that no significant harm is posed to healthy animals, whereas a THI of 75 to 78 indicates alarming thermal stress, potentially delaying weight gain. A THI of 79 to 83 indicates dangerous thermal stress, significantly reducing weight gain, and the transportation or overcrowding of water buffaloes can lead to a severe impact. A THI of ≥84 indicates an emergency state, where immediate changes are required in management to prevent endangering the animals' lives [5,17]. In the summer season (August) of 2022, the THI values ranged between 79 and 84, indicating unfavorable conditions for the buffaloes' well-being.

The average respiratory rate, rectal temperature, and HTC of the experimental buffaloes were measured in the winter and summer seasons of 2022. The respiratory rate was higher in the summer season than in the winter season, but the difference was not statistically significant. Conversely, the rectal temperature was significantly higher in winter than in summer ($P < 0.05$; Table **3**). The respiratory rate is influenced by multiple factors, such as the size and age of the animal, its physical activity level, anxiety, pregnancy, health condition, body posture, and the surrounding environmental temperature [18]. Previous studies have indicated that in summer, when the environmental temperature and solar radiation reach their peak, the rectal temperature cannot be maintained

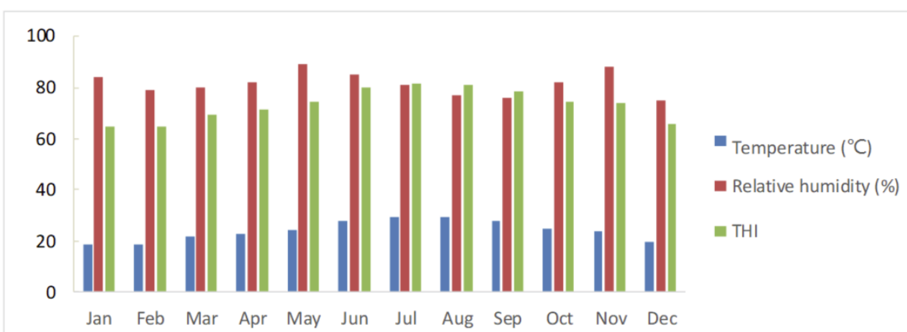

Figure 1: Average temperature, humidity, and THI in Hualien in 2022.

Table 2: Average Temperature, Humidity, and THI in the Barn of Taiwan Swamp Buffaloes in the Winter (February) and Summer (August) Seasons in 2022

Seasons	Ambient temperature (°C)	Ambient Humidity (%)	THI value
Winter(Feb)	20.05 ± 3.18^{b}	70.95 ± 3.89^{a}	66.41 ± 4.60^{b}
Summer(Aug)	28.70 ± 0.30^{a}	69.85 ± 13.93^{b}	79.38 ± 1.52^{a}

Superscripts ^{a} and ^{b} indicate significant differences in the mean values within the same column ($P < 0.05$).

Table 3: Average Respiratory Rate, Rectal Temperature, and HTC of 20 Taiwan Swamp Buffaloes in the Winter (February) and Summer (August) Seasons in 2022

Seasons	RR (breaths/min)	RT (°C)	HTC
Winter (Feb)	26.20 ± 8.40	38.70 ± 0.85[a]	2.38 ± 0.14
Summer (Aug)	29.30 ± 10.67	38.27 ± 0.29[b]	2.25 ± 0.28

Data are presented as mean ± SD for each season.
The average THI for 5 consecutive days in the winter season was 66.86 ± 4.05, and that in the summer season was 82.30 ± 4.00.
Superscripts [a] and [b] indicate significant differences in the mean values within the same column ($P < 0.05$).

within the normal range in young buffaloes. Their respiratory rate increases by five to six times, they protrude their tongues, and they increase saliva secretion [19]. In this study, the higher average rectal temperature observed in the winter season may be attributed to the presence of shade canopies and flowing water in the experimental area, allowing the buffaloes to find cool spots to decrease their body temperature.

Comparing the physiological values of the experimental buffaloes in Groups A, B, and C in the winter and summer seasons, Group A (buffaloes aged < 1 year) exhibited significantly higher respiratory rates, rectal temperatures, and HTCs in the winter season as well as higher rectal temperatures in the summer season compared with Group C (female buffaloes aged 14 to 20 years) ($P < 0.05$; Table **4**). This result indicates that buffaloes aged <1 year have greater physiological responses to environmental changes than adult buffaloes. Osei-Amponsah *et al.* [20] analyzed 150 lactating Holstein cows and selected the 20 most heat-sensitive and 20 most heat-tolerant cows based on the differences in the respiratory rate, surface temperature, panting score, and milk production. The results revealed that older cows have superior reproductive performance, feed efficiency, and heat tolerance relative to younger cows. Gupta *et al.* [21] reviewed studies on the impact of heat stress on dairy cows of different ages. In pre-weaned calves, the thermoneutral zone is an environmental temperature of 10-26 °C; beyond 26°C, all energy is used to maintain the body temperature. In growing heifers, physiological values such as the respiratory rate and rectal temperature increase at environmental temperatures of ≥24°C, and after 1 hour of exposure to 42°C, the *HSP70* concentration increases threefold. Lactating cows in summer experience increased somatic cell counts, decreased milk yield, and reduced milk quality; dry cows under heat stress have a high risk of uterine infections. These findings highlight that managing heat stress is crucial at all stages of livestock rearing, and the animal age may affect heat tolerance. In this study, a comparison of the HTCs of buffaloes across three age groups in the summer season revealed a correlation coefficient of -0.45, indicating a moderate negative correlation. This finding suggests that younger buffaloes have greater responses to heat stress.

Effects of Environmental Changes in Winter and Summer Seasons on Plasma ELISA Expression Levels of HSP70 and HSP90 in Taiwan Swamp Buffaloes

The analysis of ELISA data for the experimental buffaloes in 2022 revealed that the expression level of

Table 4: Average Respiratory Rate, Rectal Temperature, and HTC of Taiwan Swamp Buffaloes Across Three Age Groups in the Winter (February) and Summer (August) Seasons in 2022

Traits[1]	A (<1y)	B (6-9y)	C (14-20y)
WRR (breaths/min)	33.12 ± 7.55[a]	23.03 ± 6.24[b]	22.55 ± 6.02[b]
WRT (°C)	39.41 ± 0.70[a]	38.07 ± 0.57[b]	38.14 ± 0.67[b]
WHTC	2.48 ± 0.03[a]	1.99 ± 0.22[b]	1.99 ± 0.27[b]
SRR (breaths/min)	30.50 ± 10.14	28.45 ± 9.26	26.05 ± 8.77
SRT (°C)	38.56 ± 0.30[a]	38.16 ± 0.42[b]	38.08 ± 0.37[b]
SHTC	2.33 ± 0.42	2.23 ± 0.41	2.13 ± 0.39

[1]WRR, WRT, WHTC represent respiration rate, rectal temperature, and heat tolerant coefficient for 5 consecutive days in the winter season, respectively; SRR, SRT, SHTC represent respiration rate, rectal temperature, heat tolerant coefficient for 5 consecutive days in the summer season, respectively.
Data are presented as means ± SD. A one-way ANOVA was used to compare the physiological values of Taiwan swamp buffaloes across three age groups within the same season (winter or summer). Superscript letters (a, b) indicate significant differences in respiratory rate, rectal temperature, and heat tolerance coefficient among age groups ($P < 0.05$).

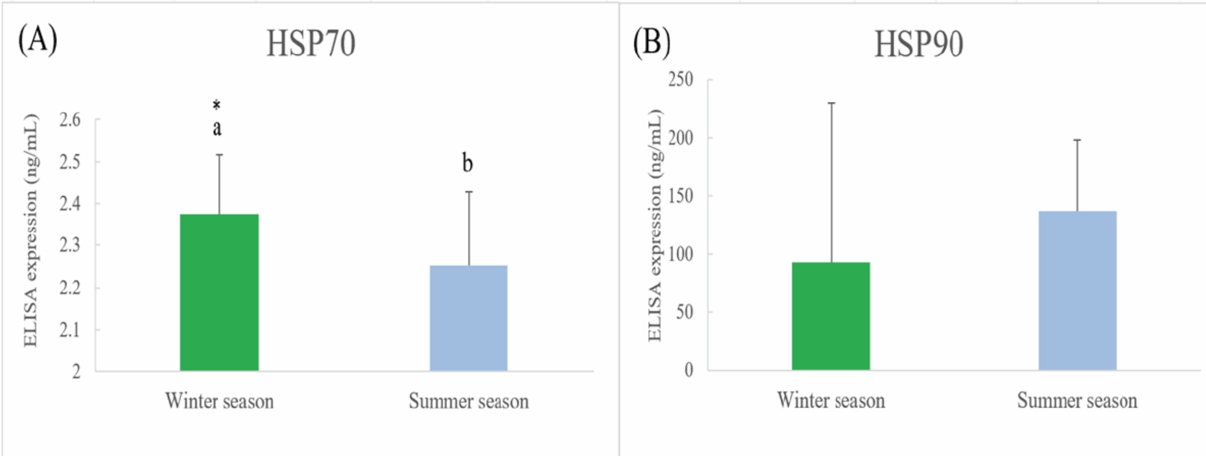

Figure 2: Plasma levels of HSP70 and HSP90 in Taiwan swamp buffaloes during the winter and summer of 2022 were measured using ELISA. Data are presented as means ± SD. In panel **A**, superscript letters (a, b) indicate significant seasonal differences in HSP70 levels, while asterisks denote significance based on an independent sample t-test ($P < 0.05$). Panel **B** shows no significant seasonal differences in HSP90 levels.

HSP70 was significantly higher in the winter than in the summer ($P < 0.05$). In contrast, the expression level of *HSP90* was higher in the summer than in the winter, though the difference was nonsignificant (Figure **2**). Large individual variation was observed, with some animals' values exceeding the instrument's detection range, resulting in missing data and a reduced sample size for statistical analysis. The mean values and standard deviations for *HSP90* expression in both seasons were calculated. Although the summer expression was higher, the difference was not statistically significant (Figure **2B**). This variability may stem from biological factors (such as age, sex, and physiological state), environmental conditions, or technical limitations during sample processing. These findings suggest a potential seasonal effect on *HSP90* expression. Previous studies indicate that *HSP90* shows signs of variation according to environmental conditions. Zeng *et al*. [22] investigated the ELISA expression levels of *HSP70* and *HSP90* in the plasma of Chinese Holstein cows under different levels of heat stress (No Heat Stress: THI < 68, Mild Heat Stress: 68 ≤ THI ≤ 79, and Moderate Heat Stress: 79 < THI ≤ 88). Under moderate heat stress, the expression levels of *HSP70* and *HSP90* were significantly higher compared to those observed under the other two stress conditions. Kumar *et al*. [23] found that in summer (April to July), the expression levels of HSP70 and HSP90 in lactating Hariana cows increased significantly at THI values of 78 and 80, respectively ($P < 0.05$). Plasma levels of HSP70 showed a single peak, reaching its maximum at THI 78, and then decreased to baseline levels, even under higher stress. In contrast, HSP90 levels peaked at THI 80 and remained elevated with further stress. These findings suggest that HSP70 provides the initial defense against heat stress, triggering the activation of HSP90 to protect the cell's structure and function. The results also highlight the interactive relationship between HSP70 and HSP90 in cellular protection under heat stress [23]. However, in this experiment, the values of some animals exceeded the instrument's detection range, reducing the statistical sample size. In the future, larger sample sizes and more controlled conditions will be necessary to confirm this observation and account for the sources of variability.

The correlation coefficient between the rectal temperature and the expression level of *HSP70*, as revealed by ELISA, in the summer season was 0.73, indicating a high positive correlation. This finding suggests that body temperature influences *HSP70* expression. However, in this study, the expression level of *HSP70* was higher in the winter season than in the summer season in 2022. The differences between the results of this experiment and previous studies can be attributed to several factors. In addition to external environmental factors, such as climatic differences due to varying latitudes and longitudes, differences in cattle breeds (dairy cows vs. buffaloes) also played a role.

Furthermore, the animals in this experiment were housed in an open barn. The buffaloes were free to choose between exposure to sunlight or retreating to shaded areas and water pools for cooling, depending on the environmental temperature and their level of discomfort. The availability of shade canopies and flowing water in the experimental area likely influenced the buffaloes' physiological parameters during summer. Notably, HSPs were first recognized as molecular chaperones due to their increased expression during heat stress. Recent studies suggest that low-temperature exposure can also trigger the transcription and translation of HSP70, highlighting their broader role in cellular stress responses [24]. HSPs are induced not only by heat shock but also by various environmental stressors, such as cold exposure, infections, hypoxia, and heavy metal presence [25]. Research has demonstrated that *HSP70* is one of the molecular chaperones associated with cold stress [26]. Studies on crustaceans [27] and mollusks [28] have examined the relationship between *HSP70* and cold stress. For example, after being transferred from low-temperature seawater to warm water, *HSP70* can be induced by cold shock in white shrimp (*Litopenaeus vannamei*). The induced *HSP70* expression scavenges reactive oxygen species, inhibits the expression of cytochrome c, prevents its release from mitochondria, and inhibits the activation of caspase-3, thereby protecting hemocytes from apoptosis caused by cold stress [25]. Kong *et al*. [24] found that in piglets exposed to 4°C for 12h, *HSP70* expression increased in the duodenum, jejunum, and ileum, indicating that *HSP70* expression can be induced by cold stress. Dangi *et al*. [29] investigated the mRNA expression of heat shock proteins (HSPs) in peripheral blood mononuclear cells (PBMCs) of goats from both tropical and temperate climates. In goats from tropical regions, *HSP70* expression was notably higher during the summer than in winter, whereas in goats from temperate regions, there was no significant seasonal variation in *HSP70* levels. In the present study, in addition to the impact of the experimental temperature on the physiological values of Taiwan swamp buffaloes, further research is required to determine whether *HSP70* expression in these buffaloes can be induced by cold stress, particularly when low-temperature warnings are issued in winter in Taiwan, as suggested by Kong *et al*. [24] and Guan *et al*. [25].

In the winter season, the expression level of *HSP70*, as revealed by ELISA, was significantly higher in Group A (buffaloes aged <1 year) than in Group B (female buffaloes aged 6 to 9 years). In the summer season, the expression level of *HSP70* (from ELISA) in Group A was significantly higher than that in Group B (female buffaloes aged 6 to 9 years) and Group C (female buffaloes aged 14 to 20 years; $P < 0.05$). However, due to large individual variations, no significant differences in *HSP90* expression levels were observed among the three groups (Figure **3**). Haque *et al*. [30] compared the average plasma *HSP70* concentrations (ng/mL) of Murrah buffaloes of different ages (1 to 2 years and 3 to 4 years) at 22°C, 40°C, 42°C, and 45°C as well as the *HSP70* levels in lymphocytes (ng/million cells) at 38°C, 40°C, 42°C, and 45°C. The results demonstrated that the plasma *HSP70* concentrations at 22°C and 45°C were significantly higher in younger buffaloes than in adult animals ($P < 0.05$). The average *HSP70* levels in lymphocyte lysates were also higher in younger buffaloes than in adults. The intensity of physiological changes was greater in younger animals than in adult

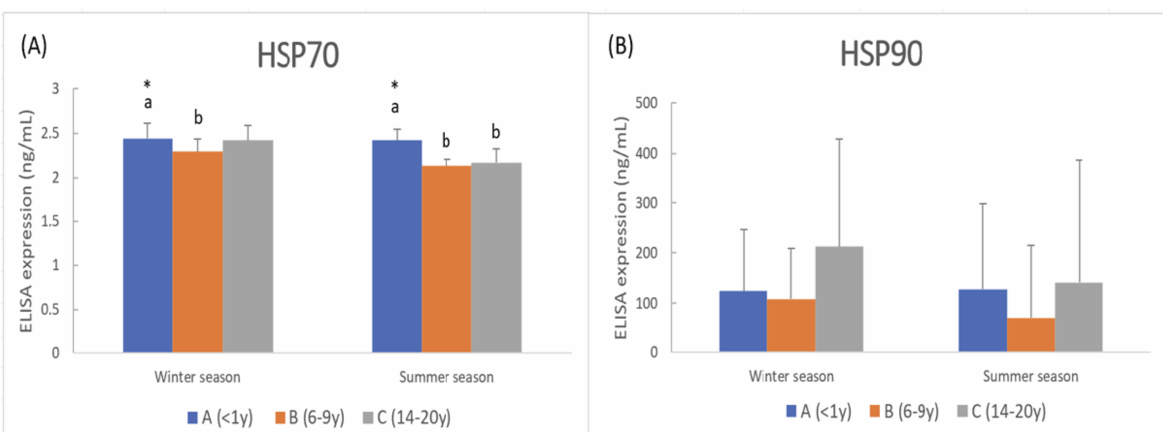

Figure 3: Plasma expression levels of HSP70 and HSP90 in Taiwan swamp buffaloes of different ages were analyzed during the winter and summer of 2022 using ELISA. Data are presented as means ± SD, and one-way ANOVA was used to compare physiological values among age groups within the same season. Asterisks indicate significant differences identified through LSD correction ($P < 0.05$). In panel **A**, superscript letters (a, b) denote significant differences in HSP70 expression levels among age groups within the same season ($P < 0.05$). Panel **B** shows no significant differences in HSP90 expression levels among age groups within the same season ($P > 0.05$).

Table 5: Genotype and Allelic Frequencies for Different SNVs in Target Gene Fragments in 20 Taiwan Swamp Buffaloes

SNV Name and Sequence	Genotype frequencies			Allele frequencies	
HSP70 g.24927355 T>C	TT(7)	TC(7)	CC(6)	T(21)	C(19)
TCATCTTTGA[T/C]CTGGGCGG	35.0%	35.0%	30.0%	52.5%	47.5%
HSP70 g.24927474 G>A	GG(8)	GA(11)	AA(1)	G(27)	A(13)
AGGCCAC[G/A]GCCGGGGA	40.0%	55.0%	5.0%	67.5%	32.5%
HSP70 exon 2 g.24928813 C>T	CC(9)	CT(7)	TT(4)	C(25)	T(15)
GTTATTT[C/T]TATATGTTA	45.0%	35.0%	20.0%	62.5%	37.5%
HSP90 g.17755706 G>A	GG(3)	GA(14)	AA(3)	G(20)	A(20)
GTCTCTGGC[G/A]GCTTTACCC	15.0%	70.0%	15.0%	50.0%	50.0%
HSP90 g.17756478 T>C	TT(8)	TC(11)	CC(1)	T(27)	C(13)
AGCAGCAAG[T/C]ACTAGCTTA	40.0%	55.0%	5.0%	67.5%	32.5%

animals, indicating the higher sensitivity of younger animals to stress. Kaushik *et al*. [31] compared the expression levels of *HSP70* mRNA in peripheral blood mononuclear cells (PBMCs) of Jamunapari goats at 3 months, 9 months, 12 months, and 2-3 years of age. During the experimental period, the environmental temperature ranged from 40°C to 49.5°C, and the Temperature-Humidity Index (THI) ranged from 82.00 to 92.08. The results showed that the HSP70 expression was highest at 9 months of age, while the expression decreased with age in the adult group. In the present study, in both winter and summer seasons, the expression levels of *HSP70* (from ELISA) in buffaloes aged <1 year were higher than those in the other two age groups, suggesting that younger buffaloes have greater responses to changes in the environmental temperature than older buffaloes.

Statistical Analysis of SNVs in Taiwan Swamp Buffaloes

We analyzed the sequence divergence of target gene fragments in the experimental buffaloes, referencing SNVs in *HSP70* and *HSP90* from a previous study [11]. The specific loci examined were *HSP70* g.24927355 T>C, *HSP70* g.24927474 G>A, *HSP70 E2* g.24928813 C>T, *HSP90* g.17755706 G>A, and *HSP90* g.17756478 T>C. Table **5** presents the genotype and allele frequencies for these loci. We investigated the polymorphisms at each site and their correlation with heat tolerance traits in Taiwan swamp buffaloes. The analysis of 20 experimental buffaloes in 2022 revealed that the buffaloes with the *HSP70* E2 g.24928813 C>T CT genotype had significantly lower respiratory rates and HTCs in the summer season than those with the TT genotype ($P < 0.05$). Additionally, the buffaloes with the *HSP90* g.17755706 G>AAA genotype had significantly lower rectal temperatures in the winter season than those with the GA genotype ($P < 0.05$).

Basiricò *et al*. [32] investigated four SNPs in the HSP70.1 gene (g895 C/− and g1128 G/T in 5'-UTR and g2154 G/A and g64 G/T in 3'-UTR) in Italian Holstein cows and their association with the heat shock response and heat tolerance of peripheral blood mononuclear cells under heat stress. The results revealed that the SNPs in the 5'-UTR region (C/− and G/T) improved the heat shock response and heat tolerance of PBMCs in dairy cows. These SNPs can serve as molecular genetic markers for selecting heat-tolerant dairy cows. Salces-Ortiz *et al*. [33] found that two SNPs (G/C2006 and A/G2444) on the HSP90AA1 gene promoter of Manchega sheep are associated with heat stress. In the present study, one buffalo from Group C (aged 14 to 20 years) possessed both the *HSP70 E2* g.24928813 C>T CT and *HSP90* g.17755706 G>A AA genotypes, both of which were found to have a significant impact on heat tolerance. This buffalo exhibited the lowest respiratory rate and HTC during the summer season. These preliminary findings suggest that the *HSP70 E2* g.24928813 C>T and *HSP90* g.17755706 G>A genotypes are associated with heat tolerance traits in Taiwan swamp buffaloes; thus, these genotypes warrant further research, and the results will serve as a reference for selecting and retaining heat-tolerant Taiwan swamp buffaloes while preserving their genetic resources.

CONCLUSION

Heat stress due to environmental temperature fluctuations concerns water buffaloes more than other cattle breeds. This study found that the physiological responses of Taiwan swamp buffaloes varied with seasonal temperature changes, with age-related differences in heat tolerance. Management practices can be adjusted for different age groups to mitigate heat stress. However, the study's scope is limited to Hualien County in eastern Taiwan, and future research should include a larger sample size and broader geographic regions to confirm these findings. Additionally, future studies could explore the genetic factors associated with heat tolerance, particularly gene polymorphisms related to heat stress. These results provide valuable insights for improving water buffalo welfare and enhancing the economic benefits of buffalo farming.

ETHICS APPROVAL

The animal housing, usage, and experimental procedures were approved by the Institutional Animal Care and Use Committee of the Eastern Region Branch of the Taiwan Livestock Research Institute (HUAIACUC11104) and were conducted in accordance with their guidelines.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest related to this study.

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