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THERMOTOLERANCE IDENTIFICATION IN WATER BUFFALO USING HEAT SHOCK PROTEIN 70 (*HSP70*) AND ITS EFFECT TO SEMEN QUALITY IN VARYING ENVIRONMENTAL CONDITIONS

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ABSTRACT

The synthesis of Heat Shock Proteins (HSPs) acts as a countermeasure for cellular protection when certain environmental parameters exceeded tolerable ranges and threatened cell survival. *HSP70*, one of the major HSPs is commonly induced in sperm of livestock animals and found to be associated with semen quality and fertility. This study aimed to determine and evaluate the role of *HSP70* in semen quality and thermotolerance of water buffalo as affected by seasonal variations. Total RNA was isolated from the ejaculates of twenty regular semen donor bulls during four seasons and was subjected to Real-time PCR for the analysis of the *HSP70* expression. The relative expression value of *HSP70* was found to be significantly (*P*<0.05) highest (1.12) during the hottest season while significantly (*P*<0.05) least (0.55) on the coldest season. A moderately positive correlation was observed between *HSP70* expression and semen quality (r=0.26-0.30). It was also found that individual bulls are capable of expressing significantly (*P*<0.05) different *HSP70* levels from 0.156-1.79. In conclusion, the expression of *HSP70* in semen can be a good indicator of thermotolerance and thermoresistance in water buffaloes as well as its semen quality, allowing it to be a potential biomarker for heat stress of an animal.

Key words: *HSP70*, heat stress, semen quality, thermotolerance, water buffalo

INTRODUCTION

Climate change is known to be adversely affecting humans and the environment. One of the most significant effect is the heat stress, which can seriously attack certain activities of the body that may alter homeostasis. Specifically, these are animal's growth, health, production and reproduction. During this time, cells have their own heat shock response through DNA damage and programmed cell death or apoptosis. This event is facilitated by a group of proteins called heat shock proteins (HSPs) (Durairajanayagam *et al*., 2014). HSPs are highly conserved proteins that are constitutively expressed in the cell and have many housekeeping

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functions. During stress, synthesis of inducible forms of HSP is enhanced and functions by protecting the cell by refolding denatured, removing damaged and degraded proteins and prevents protein aggregations (Chen *et al*., 2011; Malyshev, 2013). The amount of HSP produced to protect the cells are greater when there is longer exposure to higher temperature (Légaré *et al*., 2004).

Sperms have more mitotic activity making them more vulnerable to heat stress (Durairajanayagam *et al*., 2014). Fortunately, they have their own way to protect theirselves when a change in body temperature was felt. Theoretically in bulls, sperms residing at the scrotum must be two to six degrees centigrade lower than the body temperature to maintain its optimum condition for production, maturation, storage, minimize DNA mutations and maintenance of its quality (Bedford, 2004). Spermatogenesis in bulls takes about 61 days inside the testes. During this time, the body is exposed to different environmental conditions like heat stress, and alterations in semen occur about two weeks after and do not return to normal up until eight weeks following the end of heat stress (Hansen, 2009). Thus, an eight-week lag period for the semen collection is needed to ensure that the sperm cells were able to return to its normal state. However, this situation is not applicable to breeding centers as semen is collected even twice a week for the cryopreservation process. This then, sacrifices the quality and quantity of semen as heat stressed bulls produced lesser volume of semen, more abnormal and aged acrosome sperms which eventually leads to decrease in fertility (Birck *et al*., 2010).

Therefore, this study is conducted to determine the expression levels of *HSP70* gene on water buffalo semen during different environmental conditions and its association to semen quality. This research is being made to establish a potential biomarker for identification of thermotolerance in water buffaloes towards an enhanced management of bulls for semen collection, cryopreservation and production purposes.

MATERIALS AND METHODS

Semen samples were collected monthly from twenty-one regular donor bulls (2 Philippine Native, 1 Brazilian, 3 Italian, 14 Bulgarian Murrah) with age ranging from six to eleven years old in the National Bull Farm of Philippine Carabao Center at Central Luzon State University (CLSU). The semen donors were selected based on its estimated breeding values (EBVs) and standards set by the Agency for the testes type and scrotal circumference assuring no to minimal anatomical difference among bulls. Sperm concentration was obtained using a spectrophotometer while the initial motility was graded subjectively.

Environmental conditions were gathered from the Department of Science and Technology – Philippine Atmospheric Geophysical Astronomical Services Association (DOST-PAGASA) data collection area/office at CLSU, Science City of Muñoz, Nueva Ecija. Months of collection were classified into four seasons, with Season 1 as the Hot-Dry (February-April), Season 2 as Hot-Wet (May-July), Season 3 as Cool-Wet (August-October), and Season 4 as Cool-Dry (November-January).

The primers designed by Manjari *et al*. (2014) for Tarai Buffalo, *HSP70* (For: 5'-CTCGTCGATGGTGCTGACCAAG–3', Rev: 5'–TCCTGTCCAGGCCGTAGGCGAT–3') and *GAPDH* (For: 5'–AGGTCATCCCTGAGCTCAACGG–3', Rev: 5'–TCGCAGGAGACAACCTGGTCCTCA–3') were adapted and used for gene amplification.

RNA extraction and isolation was done following the protocols of Rajoriya

et al. (2014) with minor modifications using heated (60°C) TRIzol ® reagent (Life Technologies™, USA). Purity of the total RNA extracted was checked using NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, USA).

Synthesis of cDNA from the isolated total RNA were done using SensiFAST™ cDNA Synthesis Kit (Bioline, United Kingdom) following the manufacturer's instruction. The reaction mixture was incubated at 25°C for 15 minutes, 42°C for 30 minutes and 85°C for 5 minutes. The cDNA was stored at -4°C until further use.

A suitable PCR condition and concentration of different components was optimized for the amplification of the *HSP70* gene sequence in buffalo spermatozoa and is seen on Table 1. Confirmation of specific PCR amplicon was done by Agarose gel electrophoresis.

Shown in Table 2 are the RT-PCR profile of *HSP70* and *GAPDH* genes used for the gene expression analysis using StepOnePlus™ real-Time PCR System Thermal Cycling Block (Applied Biosystems, USA).

The amplification (Quantification cycles) and denaturation data (Melt curve), including the levels of mRNA expression were acquired and analyzed.

The equation used in determining the relative expression of *HSP70* was the 'Efficiency Calibrated Mathematical Method for the Relative Expression Ratio in Real Time PCR'. The complete equation is as follows:

$$ratio = \frac{E_{ref}{}^{CP(sample)}}{E_{target}{}^{CP(sample)}} \div \frac{E_{ref}{}^{CP(calibrator)}}{E_{target}{}^{CP(calibrator)}}$$

Where:

E_{ref} = efficiency for primer of reference gene
E_{target} = efficiency for primer of target gene
CP (sample) = Ct value of sample
CP (calibrator) = Ct value of calibrator

The means of the *HSP70* levels, initial motility and sperm concentration between bulls and seasons were calculated, as well of the different environmental parameters. Analysis of variance was used to compare means and Tukey's Post Hoc test for their significant difference at 0.05 level using SPSS Statistics Data Editor version 19.0 (SPSS Inc., Chicago, IL, USA).

Table 1. Thermal cycler profile of *HSP70* gene amplification for gene sequencing.

Thermal Profile	Time	No. of Cycles	Comments
95˚C	5 minutes	1	Initial Denaturation
95˚C	15 seconds	40	Amplification
68˚C	1 minute		
72˚C	30 seconds		
72˚C	10 minutes	1	Final Extension

Table 2. Thermal cycler profile of *HSP70* and *GAPDH* on real-time PCR.

Gene	Segment	Thermal Profile	Time	No. of Cycles	Comments
HSP70	Segment 1	95˚C	15 minutes	1	Initial Denaturation
	Segment 2	95˚C	15 seconds	40	Real-Time Amplification
		68˚C	1 minute		
	Segment 3	95˚C	1 minute	1	Melt Curve Analysis
		60-90˚C	30 seconds		
		95˚C	30 seconds		
GAPDH	Segment 1	95˚C	15 minutes	1	Initial Denaturation
	Segment 2	95˚C	15 seconds	40	Real-Time Amplification
		65˚C	1 minute		
	Segment 3	95˚C	1 minute	1	Melt Curve Analysis
		60-90˚C	30 seconds		
		95˚C	30 seconds		

RESULTS AND DISCUSSION

The study analyzed the *HSP70* gene from the semen of the different breeds of water buffaloes using end-point polymerase chain reaction and was confirmed by subsequent agarose gel electrophoresis (AGE) (Figure 1). Samples showing positive results after AGE were sent for sequencing to confirm the validity and efficiency of the primers used.

The partial sequences of the different breeds of water buffalo analyzed encompassed 206bp and were 100% identical among all breeds. Also, it was found out that the bubaline *HSP70* has 97-99% homology with bovine, caprine and swine *HSP70* based on the Basic Local Alignment Search Tool (BLAST) of the National Center for the Biotechnology Information (NCBI) database, as presented in Figure 2.

Real time PCR was optimized for the *HSP70* relative expression. Quantification cycles (Qc) of different samples were noted for data analysis as shown on the amplification plot (Figure 3a). The *HSP70* gene amplified from each samples were validated by its characteristic melting curve (Figure 3b), which showed only one peak.

The means of the environmental parameters experienced during the different seasons of the year (2016) were shown on Table 3. The highest mean temperature recorded during Season 1 is significantly higher ($P<0.05$) than the other three seasons. The significantly highest ($P<0.05$) mean relative humidity, on the other hand, occurred during Season 2 while Season 4 had the significantly lowest ($P<0.05$). It is also worth noting that the computed THI of all the seasons, with values greater than 25.6, are what Marai *et al*. (2001) considered as extreme severe heat stress for cattle and buffalo alike. With that, the worst heat stress experienced by the animals in this paper was during Season 1 which is significantly the highest THI ($P<0.05$).

The difference in the *HSP70* relative expression level, initial motility and sperm concentration as factors affecting semen quality during different seasons are presented in Figure 4. The significantly highest ($P<0.05$) mean relative expression values of *HSP70*

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Bubaline    TCTCGTCGATGGTGCTGACCAAGATGAAGGAGATCGCGGAGGCGTACCTG
Bovine      TCTCGTCGATGGTGCTGACCAAGATGAAGGAGATCGCCGAGGCGTACCTG
Caprine     TCTCGTCGATGGTGCTGACCAAGATGAAGGAGATCGCCGAGGCGTACCTG
Swine       TCTCGTTGATGGTGCTGACCAAGATGAAGGAGATCGCGGAGGCGTACCTG

Bubaline    GGCCACCCGGTGACCAACGCGGTGATCACCGTGCCGGCCTACTTCAACGA
Bovine      GGCCACCCGGTGACCAACGCGGTGATCACCGTGCCGGCCTACTTCAACGA
Caprine     GGCCACCCGGTGAGCAACGCGGTGATCACGGTGCCGGCCTACTTCAACGA
Swine       GGCCACCCGGTGACCAACGCGGTGATCACCGTGCCGGCCTACTTCAACGA

Bubaline    CTCGCAGCGGCAGGCCACCAAGGACGCGGGGGGTGATCGCGGGGCTGAAC
Bovine      CTCGCAGCGGCAGGCCACCAAGGACGCGGGGGGTGATCGCGGGGCTGAAC
Caprine     CTCGCAGCGGCAGGCCACCAAGGATGCGGGGGGTGATCGCGGGGCTGAAC
Swine       CTCGCAGCGGCAGGCCACCAAGGACGCGGGGGGTGATCGCGGGGCTGAAC

Bubaline    GTGCTGAGGATCATCAACGAGCCCACGGCCGCCGCCATCGCCTACGGCCT
Bovine      GTGCTGAGGATCATCAACGAGCCCACGGCCGCCGCCATCGCCTACGGCCT
Caprine     GTGCTGCGGATCATCAACGAGCCCACGGCGGCGGCCATCGCCTACGGCCT
Swine       GTGCTGAGGATCATCAACGAGCCCACGGCCGCCGCCATCGCCTACGACCT

Bubaline    GGACAGGAA
Bovine      GGACAGGAC
Caprine     GGACAGGAC
Swine       GCACAGGAC
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Figure 1. Alignment of *HSP70* partial sequences of different representative mammalian species.

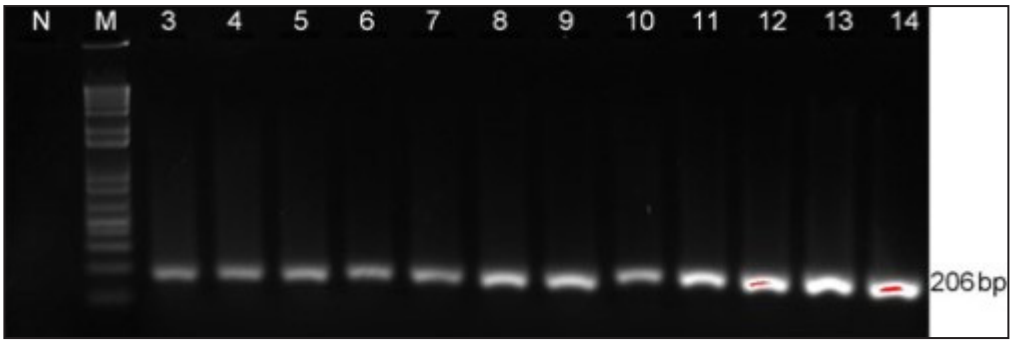

Figure 2. Amplification of *HSP70* gene by RT-PCR.
Lane N- Negative Control; Lane M- Molecular Ladder Marker (1kb+);
Lane 3-14- 206bp HSP70 bands.

was observed during Season 1 (1.12) and is approximately two-folds higher than the significantly lowest (P<0.05) value of 0.55 from Season 3. In terms of the sperm concentration, Season 2 has the significantly highest (P<0.05), 119.92 million sperms/ml, while Season 4 has the significantly least (P<0.05), 94.40 million sperms/ml. For the initial motility, there is no significant difference among the seasons except for Season 2 which has the significantly least (P<0.05) of 59.21%.

A correlation was made for the *HSP70* relative expression, initial

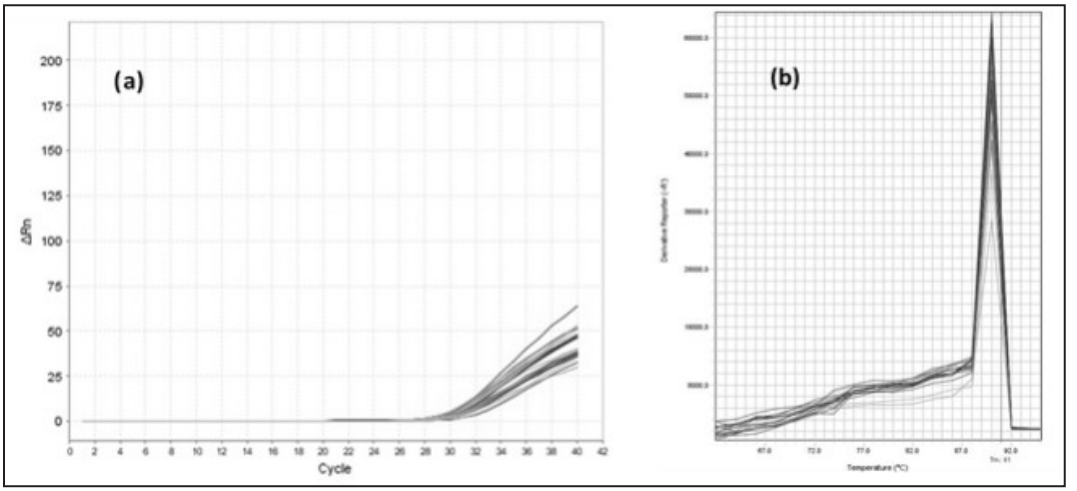

Figure 3. Amplification Plot (a) and Melt Curve (b) of *HSP70*

motility and sperm concentration with the different environmental conditions as grouped by seasons. A strong positive correlation was observed for the temperature (r=0.60), strong negative correlation for relative humidity (r=-0.60) and moderately positive correlation with THI (r=0.24). For the sperm quality, a moderately positive correlation was observed for both the initial motility (r=0.30) and concentration (r=0.26). From these, it can be inferred that the temperature and RH are highly affecting the *HSP70* relative expression level. While both the increase in temperature and decrease in RH can cause an elevation of the *HSP70* relative expression level. Meanwhile, increasing values for THI can bring an escalation in the *HSP70* relative expression level. On the other hand, both the initial motility and sperm concentration was affected positively as the *HSP70* relative expression level increases.

Furthermore, we can also observe a negative correlation between the RH and the initial motility (r=-0.22) and sperm concentration (r=-0.18). Apparently, a low to almost no correlation for the temperature and semen quality was observed. By this, we can infer that the RH as an environmental parameter considerably affects the semen quality.

The mean relative expressions of *HSP70* are shown in graph on Figure 5 to outline the differences of individual bulls. The significantly highest (P<0.05) *HSP70* relative expression level was produced by Bull 7 (1.79) which is approximately two- to nine-folds higher than the rest of the animals. It is also worth noting that Bull 14 and 15 (0.157 and

Table 3. Environmental parameters under different seasons.

Season	Description	Mean Temperature (˚C)	Mean Relative Humidity (%)	Temperature-Humidity Index (THI)
1	Hot-Dry	28.88[a]	74.51[b]	27.7[a]
2	Hot-Wet	27.38[b]	85.83[a]	26.8[b]
3	Cool-Wet	27.17[b]	75.52[b]	26.2[c]
4	Cool-Dry	27.33[b]	70.61[c]	26.1[c]

*Means within the same column with different letters differ significantly (P<0.05)

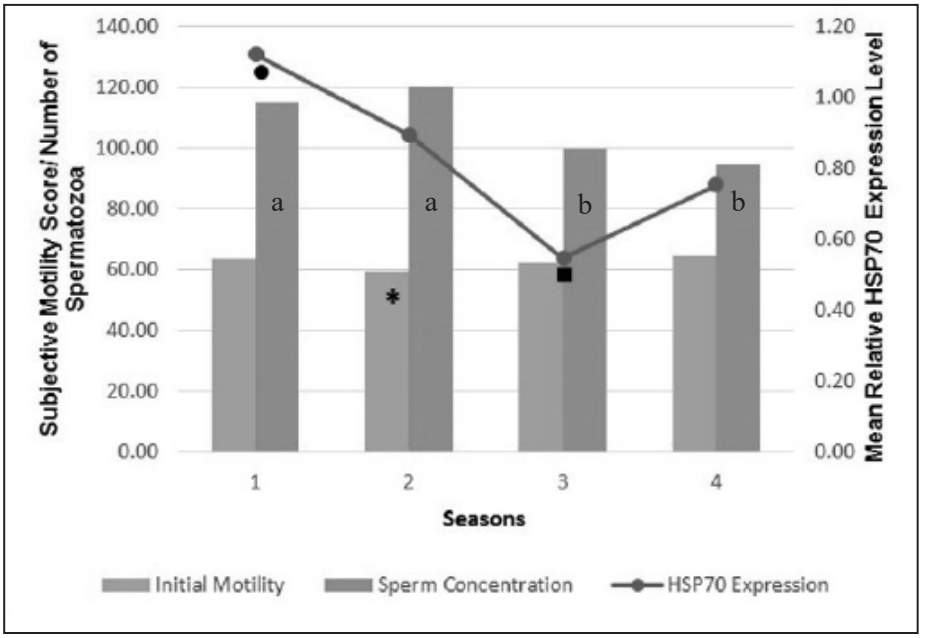

Figure 4. Association of semen quality and gene expression level of *HSP70* across seasons. Bars of the same color with the different letter indicate difference at 0.05 level of significance. Bar(s)/ Point(s) with the significantly lowest values are denoted with asterisk (*) and a square (■) while those with significantly highest values is represented with circle (●).

0.156, respectively) gave the significantly least (P<0.05) *HSP70* relative expression level which is only a tenth of Bull 7. The significantly different mean relative values of *HSP70* among bulls firmly indicate a variation on their response to heat stress and may be a potential marker in determining animal's thermotolerance and thermoresistance.

In mammals, the cellular response to thermal stress is controlled at the transcription level and mediated by the heat shock transcription factors which are regulated by the inducible expression of HSF genes. Among these HSF genes, *HSP70* is the most identified ideal biomarker for heat stress in farm animals, particularly in cattle (Gafer *et al*., 2015; Zhang *et al*., 2015), goat (Choi *et al*., 2013), boars (Huang *et al*., 2000) and some in buffaloes (Manjari *et al*., 2014; Zhang *et al*., 2015).

In this study, positive correlations were observed between *HSP70* level and the initial motility and sperm concentration. Similar findings were noted between the correlation of initial motility and *HSP70* levels in bovine bulls, 0.327 (Muiño *et al*., 2009) and in boars, 0.00-0.14 (Huang *et al*., 2000). Furthermore, expression of *HSP70* across varying seasons were found to be significant from each other and positively affecting initial motility. Comparable in this paper were the results of Huang *et al*. (2000) in cattle with an increasing amount of *HSP70* during hot season (1.0-1.8) and with Manjari *et al*. (2014) in Tarai buffalo (greater than 1.0). Contradicting to that were the results in Tharparkar bull with no significant difference recorded with the *HSP70* levels in summer and winter season, although a numerically higher *HSP70* was recorded during summer (Rajoriya *et al*., 2014). This escalation during hot or summer conditions were agreed to be due to the protective

action of HSPs on the cellular auto-regulation in response to heat and on the mechanism of homeostasis, providing a balance between protein synthesis and degradation (Basiricò *et al*., 2011). *HSP70* expression in the cells restrained the activity of protein kinase including p38 and Jun N-terminal kinase (JNK), which strengthened the cell resistance. Relating to the semen quality, the increase of *HSP70* level expression restrained ATP degradation, thus sperm had sufficient energy to express more *HSP70* and maintain motility after. In addition, *HSP70* could activate the activity of Ca^{2+} ATPase which was being inhibited by the stress brought about by heat, thus, reduce membrane damage, and relieve the effect of stress reaction in the mitochondria, allowing the ATP to be utilized for the sperm movement (Mishra *et al*., 2013; Zhang *et al*., 2015).

In this study, significant differences on the *HSP70* level on different seasons and on semen quality, suggests that it can be a valuable criterion of thermotolerance and thermoresistance. The optimum temperature for growth and reproduction in buffaloes is only between 13-18°C and 55-65% relative humidity (Ahmad *et al*., 2013; Manjari *et al*., 2014), any higher than this may already be considered as stressful for water buffaloes (Marai & Haeeb, 2010). Heat resistance in animal is defined as the innate characteristic while thermotolerance, on the other hand, is only a transient ability of an animal resulting from various interplaying factors (physiological, behavioral, cellular, etc.). Expression of *HSP70* is only one of the many ways by which animals are able to acquire thermotolerance and the varying level found between animals can contribute to their different thermotolerance level (Pawar *et al*., 2014; Choi *et al*., 2013; and Huang *et al*., 2000). Singh *et al*. (2014) concluded that heat resistant animals have a large amount of constitutive HSP70 (HSC70) in their

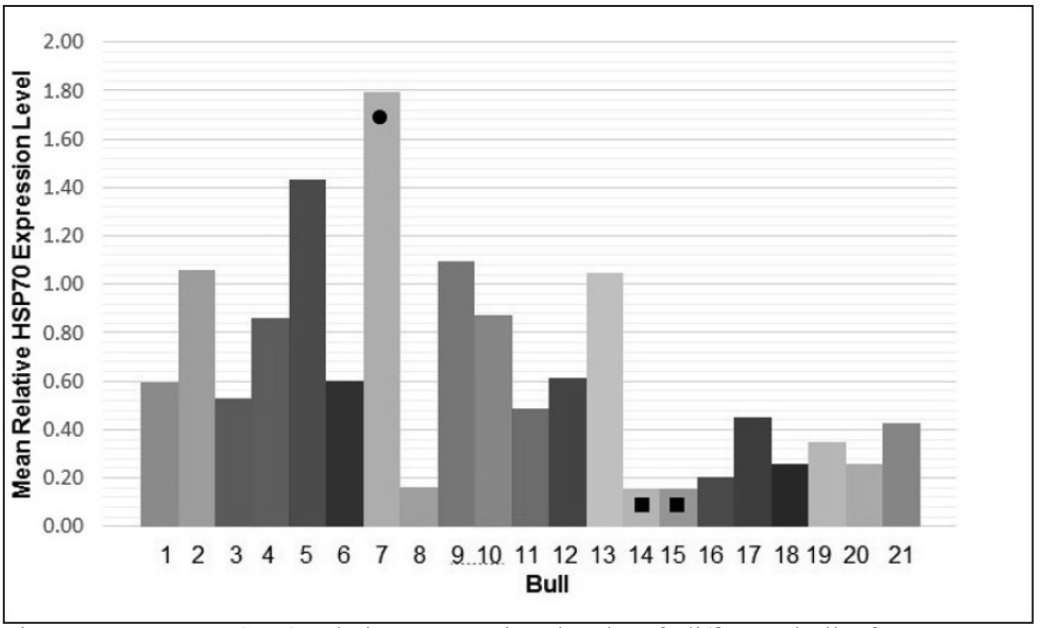

Figure 5. Mean *HSP70* relative expression levels of different bulls for one year. Bar(s)/Point(s) with the significantly lowest values are denoted with a square (■) while those with significantly highest values is represented with circle (●).

system consequently limiting the synthesis of additional HSP70. With that, less resilient animals have less constitutive HSP70 and are capable of producing more HSP70 upon heat stress. The high level of HSP70 allows them to develop thermotolerance for a limited time. Contrasting levels of HSP70 between individuals may have arisen from the differences in the animals' mRNA splicing, transport and stabilization mechanisms. Another possibility could be the variation on the transcriptional (Schiaffonati and Tiberio, 2008) or on post-transcriptional regulation (Hunter-Lavin *et al*., 2004) of gene expression of individual animals. The accumulation of damaged or damaged proteins in the cell after stress exposure, perhaps due to the insufficiency of constitutive HSP70, is highly likely to attenuate the expression of inducible HSP70 for protein refolding and cell repair. Reinforcing the fact are the reports demonstrating that lower expression of HSP70 could be an indication of high heat resistance (Choi *et al*., 2013 and Howard *et al*., 2014) of the animal.

The findings of this study indicates that the high HSP70 level found in bulls can suggest a high thermotolerance whereas the minimal amount expressed can be a sign of heat resistance. Finally, this strengthens the identification of expression of *HSP70* in semen to be used as a potential biomarker for thermotolerance in water buffaloes, which will lead to the recognition of ideal environmental and management conditions for an enhanced semen quality production, growth and over-all development of the animal.

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