



Study on the effect of seasonal variation on expression of serum biochemicals in pigs

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ABSTRACT

Pigs are particularly susceptible to heat stress because of their more sub-cutaneous fat with little or non-functional sweat glands. Pigs depend on panting as primary mechanism of heat dissipation and/or wallowing. Therefore, a study was conducted to generate basic information on certain blood biochemicals of pigs reared in high humid climatic condition. For the purpose, blood samples were collected randomly from 400 female pigs (6–12 month of age) during summer and winter of the year. The analysis of THI has shown an increase in the study area since 2012 to 2018. The hematological study of the pigs revealed a variation in respect of Hb and TEC with the season. Estimation of different enzymes showed increase with the increase of THI. The activity of enzyme, hormones except leptin was accelerated. Expression of IGF 1, HSP 70 and HSP 90 was also affected with the change of THI. The level of Kisspeptin and FSH β receptor showed difference between two seasons of the year. However, the level of Kisspeptin and FSH β receptor in serum of the experimental animals was found to be insufficient to initiate puberty till 12 months of age. It can be concluded that the pigs reared in semi-intensive system under high humid climatic condition did not attain puberty up to one year of age.

Keywords: Enzymes, Heat stress, Hormones, Swine

Heat stress is one of the major concerns in pig, the two most important tools (sweating and panting) for the maintenance of body temperature is less developed. Pigs do not have functional sweat glands to remove body heat efficiently and have relatively small lungs size for efficient panting. Further, their relatively thick sub-cutaneous fat makes the pigs more prone to heat stress. It was also reported that heat stress can create secondary infection in pigs as their intestinal defence systems are significantly compromised and this provides opportunity for infection as pathogenic bacteria can invade the body more easily. Therefore, heat stress can create secondary infection if sanitary conditions are poor (Pearce *et al.* 2013). In general, pigs try to minimize the effects of heat stress by two major methods. These include an increase in heat dissipation and a reduction in heat production from body metabolism. To increase heat dissipation, pigs will increase their body surface area by sprawling out to increase contact with a cool surface like a floor (Pourouchottamane *et al.* 2013). The climate change scenario alarms the significant probable impact on pig farming system in Assam (Soren *et al.* 2018). Among the different agro climatic zone of Assam, the North

Blank Plain zones has comparatively higher pig population and are primarily reared by low input traditional semi-intensive system. Locally available feed resources are the main source of nutrition of the pigs. The climate in the area of study have moderate to high temperature with higher relative humidity. The information on blood biochemicals of pigs reared in humid conditions is scanty. Therefore, the present study was conducted to generate basic information on certain biochemicals related to thermal, nutritional stress and reproduction of pigs reared in high humid condition of North Blank Plain zone of Assam.

MATERIALS AND METHODS

Animals and blood sampling: Gilts (400) of six months age, reared under semi-intensive system were randomly selected from the North Bank Plain zone of Assam. The climate of North Bank Plain zone of Assam is sub-tropical between longitudes 20°09'N to 26°95'N and latitudes 91°45' E to 92°22' E. Blood samples were collected from each of the experimental animals during summer (April to August) and winter (December to February) in the morning hours.

Collection of meteorological data and calculation of Temperature humidity index (THI): Meteorological data of Lakhimpur district were collected from the Regional Agricultural Research Station, Assam Agricultural University, North Lakhimpur, Assam. The data of Dhemaji district were collected from the Meteorological and

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Oceanographic Satellite Data Archival Centre. The temperature humidity index (THI) was calculated by the method of Johnson *et al.* (1962) and Ravagnolo and Misztal (2000). The level of THI between 65 and 72 was considered comfortable, THI between 72–80 is considered as mild stress, 80–90 as medium and 90–98 as severe stress (Upadhyay *et al.* 2007).

Hematological parameters: Haemoglobin (Hb) and packed cell volume (PCV) were estimated by acid hematin and micro hematocrit method, respectively. The total erythrocyte count (TEC) and total leukocyte count were estimated by Hemocytometer method.

Estimation of biochemical parameters: SGOT, SGPT, LDH and ALP were estimated by commercially available kit (Siemens Healthcare Private Limited, Gujarat, India) using spectrophotometry method as per the manufacturer protocol. Kisspeptin, FSH β receptor, T₃, T₄, Leptin, Ghrelin and Cortisol were estimated using commercially available ELISA kits as per manufacturer protocol.

Total RNA isolation and cDNA synthesis: The total RNA was isolated using ZR whole blood RNA miniPrep kit (cat. No. R1020) from Zymo research as per the method given by manufacturer. Briefly, 600 μ l blood RNA buffer was added to 200 μ l of blood just after collection from animals. The mixture was transferred to zymo-spin IIIc column and centrifuged for 1–2 min at 10,000 rpm. The mixture content was also passed (after 2–3 wash) through zymo-spin IC column for yielding higher concentration of RNA. Finally, RNA was eluted in a DNase/RNase free eppendorf tube. About 300 ng of total RNA was used for reverse transcription-polymerase chain reaction (RT-PCR) using Revert Aid First strand c-DNA synthesis kit (Fermentas, USA) according to manufacturer's protocol. Briefly, the mixture of RNA and oligo (dt) primer, 5 $\times$ reaction buffer, Ribolock RNase inhibitor (20 U/ μ l), 10 mM dNTP mix and revertAid M-MuLVRT (200 U/ μ l) were added to a 0.2 ml sterile tube and made it to 20 μ l by adding nuclease-free water. The RT-PCR was carried out at 65°C for 5 min, 42°C for 60 min and 70°C for 5 min in a thermocycler (Verti, Applied Biosystem). The c-DNA samples were stored at –20°C after diluting to 1 : 1 ratio.

Semi-quantitative PCR: Semi-quantitative PCR (qPCR, Applied Biosystems®7500 Real-Time PCR) was used to analyze the relative expression of candidate genes. The annealing temperatures for all the primers (Table 1) were evaluated through gradient PCR (Verti, Applied Biosystem), amplification of candidate genes were confirmed by

observing the product size (using 2.5% agarose) under a Gel documentation system (E-gel image, life technology). The semi-quantitative PCR reaction was carried out using Maxima SYBR green real-time PCR (qPCR) mAXer mix (10 μ l) along with forward and reverse primers (1 μ l, 10 pmol), nuclease free sterile water (7 μ l) and template (1 μ l). Negative controls were run in each PCR assay without template (cDNA). The PCR product of candidate genes was confirmed (2.5% agarose) by observing in Gel documentation system. The qPCR program consisted of initial heating at 50°C for 2 min followed by 95°C for 10 min, annealing (Table 1) for 60 sec, and amplified for 40 cycles. The final extension at 72°C incubation was continued for a further 10 min.

Statistical analysis: The data of hematological (Hb, PCV, TEC, TLC) and biochemical (SGPT, SGOT, ALP, LDH, T₃, T₄, Leptin, ghrelin and cortisol) parameters during summer and winter were analyzed by student 't' test using MS-Excel software. The relative expression level of candidate genes (HSP70, HSP90 and IGF-1) was calculated by comparing the expression level of reference gene, i.e. GAPDH as per the method Livak and Schmittgen (2001). The gene expression of candidate genes during summer and winter was also analyzed by student 't' test. Graphs were plotted using Prism 5 software.

RESULTS AND DISCUSSION

The calculated THI revealed an inclement weather for the productive and reproductive performances of pigs during April to October since 2012–18. It is well described that when THI exceeds from 72, the productive and reproductive performances can be affected in farm animals. Moreover, pigs lack a well-developed cutaneous thermoregulatory mechanism; it makes them more uncomfortable with the changing environment. The present study revealed that the THI from April to October in last seven years (2012–18) was >70 and it may oscillate in near future. THI is considered as an indicator of thermal climatic conditions. Mild, medium, and severe stress was classified on the basis of THI range, i.e. 72–80 (mild), 80–90 (medium), and 90–98 (severe). Livestock species are comfortable at THI between 65 and 72. The THI of pig populated area (study area) of Assam is more than 80 during the months from June to September (2012–18). THI during 9:30–16:30 (24 h) is increasing from 2012 to 2018, where animals generally do their activities. However, the THI during their resting time (20:30–5:30) also increased slightly

Table 1. The sequence of forward and reverse primers of target genes, their amplicon size (bp) with annealing temperature.

Gene symbol	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Amplicon Size (bp)	Annealing Temperature (°C)	References
HSP 70	ACAACCAGTTGGCGGAGAAA	GAGCTTGAGCACCACAACCTG	132	60.0	Vashi <i>et al.</i> 2018.
HSP 90	CGCTGAGAAAGTGACCGTTATC	ACCTTTGTTCCACGACCCATAG	126	60.0	Kamanga-Sollo <i>et al.</i> (2011)
GAPDH	CACTGAGGACCAGGTTGTGT	GCTTGACGAAGTGGTCGTTG	101	60.0	Vashi <i>et al.</i> 2018.
IGF1	TCTTCTACTTGGCCCTGTGCTT	CCAGCTCAGCCCCACAGA	80	60.5	Slifizier <i>et al.</i> 2013

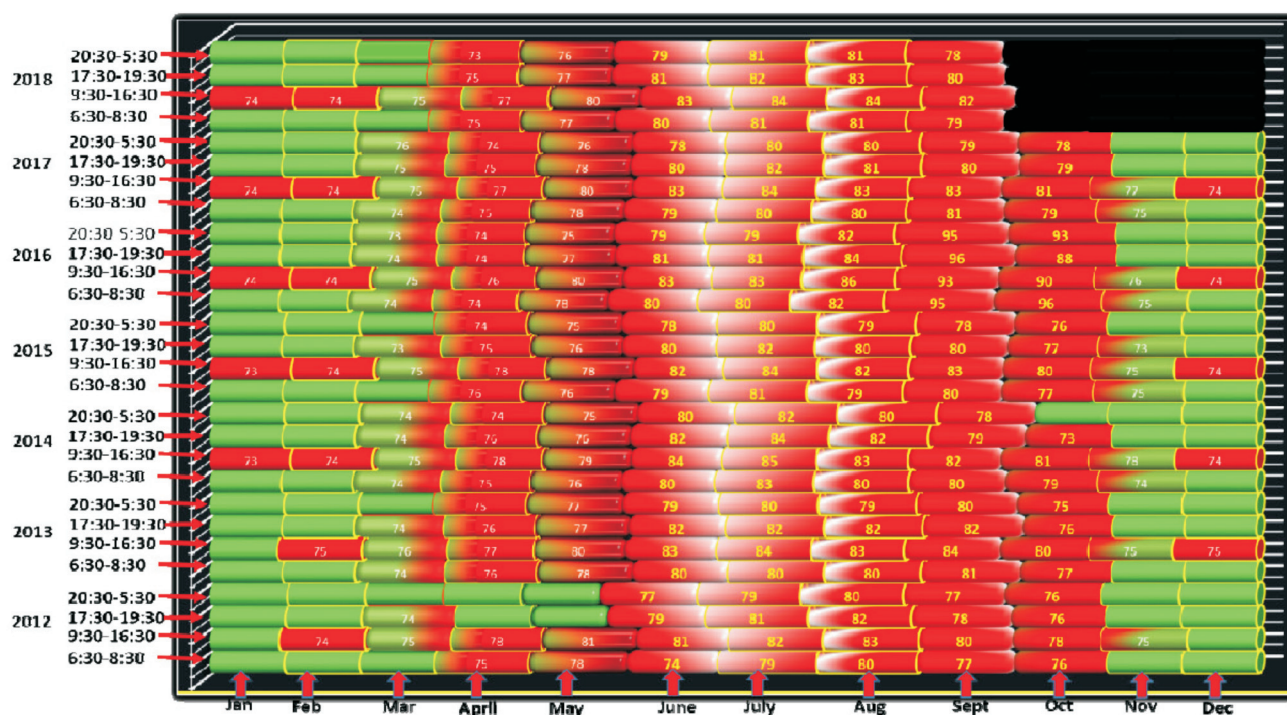


Fig. 1. Temperature humidity index (THI) of last five year during different time of 24 h periods.

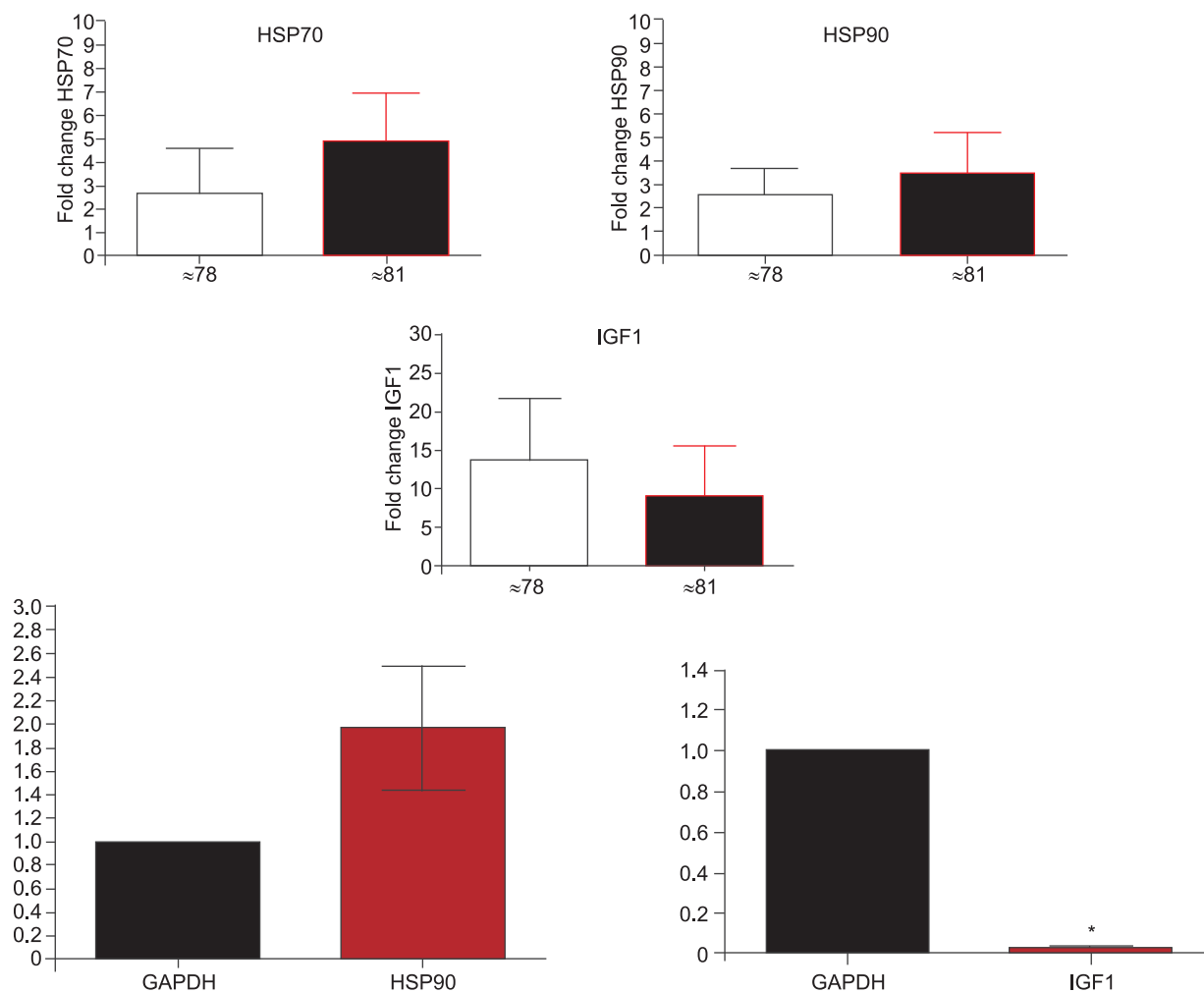


Fig. 2. The expression profile of HSP70, HSP90 and IGF1 gene at different discomfort zone (THI ~78 & 81).

from 2012 to 2018 (Fig. 1.). The trend of increasing THI during different hour of a day indicates the stress level that may impact on piggery production. THI above 72 was also seen during the months of January, February, March, November and December at some hours of a day. The comparison of THI from 2012 to 2018 revealed an average increase of THI during last four years in the study area.

Haematological parameters: In the present study the PCV and TLC are not affected by season, however, Hb and TEC were found to be higher ($P<0.01$) in winter season than in summer (Table 2). The haematological values in the present study were within the normal reference range for pigs (Schalm *et al.* 1986). Similar results of seasonal variation in Hb, TEC was reported earlier (Mayengbam and Tolengkomba 2015). The lower Hb and TEC in summer might be due to voluntary reduction in feed intake during and impaired absorption of nutrient from the intestinal tract (Pearce *et al.* 2013).

Effect of thermal stress on serum enzyme concentration of pigs: The average SGPT and SGOT level in pigs were higher ($P<0.01$) during summer (57.15 ± 0.19 U/L and 48.06 ± 0.09 U/L) than in winter (43.96 ± 0.11 U/L and 37.78 ± 0.06 U/L) (Table 2). The ALP and LDH were higher ($P<0.01$) during summer season (27.85 ± 0.03 KAU/100 ml and 379.00 ± 0.15 IU/L) than in winter (18.77 ± 0.03 KAU/100 ml and 353.40 ± 0.27 IU/L) (Table 2). The higher concentration of enzymes might be attributed from inclement weather (THI>70). This finding is in corroboration with Marai *et al.* (1995) in calf, Chetia *et al.* (2017) in pigs. ALP activity of adult pigs was lower in summer than in rainy and winter seasons, which might be due to summer stress as reported earlier (Devgun *et al.* 1998).

Effect of season on metabolic hormonal profile of pigs: The lower ($P<0.05$) concentration of T_3 and T_4 was estimated during summer season indicates a compromise in metabolism to reduce the internal heat production. Thyroid hormone is an indicator of metabolic status of an animal (Magdub *et al.* 1982, Bhattacharya *et al.* 1994, Bhattacharya *et al.* 1995, Dutta *et al.* 2002 and Bhooshan *et al.* 2010), it is also a suitable indicator of thermal stress

Table 2. Blood biochemical parameters of experimental animals

Parameter	Summer	Winter
Hb (g/dl)	$11.81^a\pm0.13$	$11.21^b\pm0.12$
PCV (%)	$33.04^a\pm0.30$	$33.01^a\pm0.31$
TEC ($\times 10^6/\mu\text{L}$)	$12.96^a\pm0.25$	$14.55^b\pm0.20$
TLC ($\times 10^3/\mu\text{L}$)	$11.71^a\pm0.17$	$11.79^a\pm0.16$
SGPT (U/l)	$57.15^a\pm0.19$	$43.96^b\pm0.11$
SGOT (U/l)	$48.06^a\pm0.09$	$37.78^b\pm0.06$
ALP (KAU/100 ml)	$27.85^a\pm0.03$	$18.77^b\pm0.03$
LDH (U/l)	$379.00^a\pm0.15$	$353.40^b\pm0.27$
T_3 (ng/dl)	$0.658^a\pm0.025$	$0.817^b\pm0.066$
T_4 (ng/dl)	$39.1^a\pm1.23$	$44.795^b\pm0.97$
Leptin (ng/ml)	$0.012^a\pm0.0008$	$0.010^a\pm0.0007$
Ghrelin (ng/ml)	$194.005^a\pm0.321$	$184.640^b\pm0.139$
Cortisol (nmol/l)	$114.31^a\pm6.86$	$94.45^b\pm5.67$
FSH β receptor (mIU/ml)	$91.72^a\pm0.25$	$71.59^b\pm0.3$
Kisspeptin (ng/ml)	$0.355^a\pm0.004$	$0.243^b\pm0.003$

(Rasooli *et al.* 2004, Abilay *et al.* 1975, West *et al.* 1999).

Effect of season on Ghrelin and Leptin concentration in pigs: The concentration of Ghrelin was higher ($P<0.01$) during summer season whereas, Leptin concentration was not affected by season. Ghrelin in pigs showed increase in energy insufficiency (Bradford and Allen 2008; Wertz-Lutz *et al.* 2010), regardless of the feeding regime (Scrimgeour *et al.* 2008). The high concentration of Ghrelin during summer might be due to the diversion of blood flow to skin surface to dissipate excess heat leading to intestinal hypoxia, oxidative and nitrosative stress in enterocytes (Lambert 2004; Pearce *et al.* 2013) resulted "leaky gut" (increase in permeability). The elevated ghrelin in summer might correspond with poor forage quality, reduced consumption and energy expenditure (Stantorf *et al.* 2017). Similarly Pearce *et al.* (2014) reported increase in concentration of ghrelin during heat stress in pig.

Effect of season on cortisol, HSP 70, 90 and IGF-1: The higher ($P<0.05$) concentration of cortisol in the study during summer indicated discomfort, also observed in growing and finishing pigs by Fagundes *et al.* (2008). The expression of HSP70 increased at THI 81 than 78 signifying the stress level leading to increase the secretion of cortisol during summer. Further, the expression of HSP90 also increased at THI 81 than 78 that might be attributed by nutritional stress as revealed higher ghrelin concentration in the study. The expression of IGF 1 decreased at THI 81 than 78 (Fig. 2) indicating the changes in systemic and intracellular energy metabolism (Fernandez *et al.* 2015) essential to adapt in a thermal challenged environment. The decreased IGF-1 might be due to improper insulin homeostasis which resulted in reduced growth (Pearce *et al.* 2013).

Effect of season on Kisspeptin and FSH β receptor: The expression of biomolecules related to reproduction showed a significant ($P<0.01$) difference between the seasons. However, the level of Kisspeptin and FSH β receptor were found to be insufficient to initiate puberty. The optimum concentration of kisspeptin is important in pre-pubertal gilt to stimulate the secretion of FSH, Estrogen for attainment of puberty followed by ovulation and secretion of progesterone from the corpus luteum (Lents *et al.* 2008, Ross, 2014). This insufficient concentration might be due to nutritional (Table 2) as well as environment stress as estimated in the present study. Pigs reared under low input system of rearing are not able to attain puberty below one year of age (data collected from farmer by personal interview). This delayed puberty in pigs in the area of study might be attributed to nutritional and thermal stress.

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