EXPRESSION OF SKIN COLOUR GENES IN THARPARKAR CATTLE DURING SUMMER AND WINTER SEASON

Maibam Uttarani, Singh S. V.,* Upadhyay R. C., Kumar Suresh, Beenam and Singh A. K.
Dairy Cattle Physiology Division, National Dairy Research Institute, Karnal, Haryana (INDIA)

Received November 20, 2013 Accepted July 22, 2014

ABSTRACT

In order to observe the expression of the genes (MC1R and PMEL) and activity of the enzyme (tyrosinase) related to skin pigmentation, study was conducted on Tharparkar heifers. Blood samples were collected from Tharparkar heifers (2-3 years) during summer (T_{max}, 36.4ºC) and winter (T_{min}, 4.1 ºC) season at weekly interval. Just after blood collection, samples were transferred to the laboratory for lymphocytes separation and isolation of RNA. The RNA samples were further processed for cDNA preparation from which the relative mRNA expression of the genes was quantified using RT-PCR. The tyrosinase activity was determined in plasma samples using Bovine tyrosinase ELISA kit. The relative MC1R and PMEL mRNA expression in lymphocytes of Tharparkar heifers was found to be significantly (P<0.05) higher during winter season than summer season. Similarly, the activity of plasma tyrosinase enzyme was also found to be significantly (P<0.05) higher during winter season. The thyroidal hormones (Thyroxine and Triiodothyronine) was found to be significantly (P<0.05) higher during winter season than summer season. Thyroidal hormones showed the positive correlation with the skin colour related genes and tyrosinase enzyme activity. The results of the present study clearly showed the significant difference in the expression levels of skin colour related genes (MC1R and PMEL) and tyrosinase activity during the two different seasons. Therefore, it can be stated that these genes and tyrosinase enzyme activity is related to adaptability of Tharparkar cattle to different ambient conditions.

Key Words : MC1R, PMEL, Tyrosinase, Thyroidal hormone, Tharparkar heifers

INTRODUCTION

Cattle are homeotherms that maintain their body temperature within a narrow range. Under stress, physiological, biochemical and morphological responses vary with animal’s genetic makeup and environmental conditions. The relationship between behavioural and physiological indicators can be used to evaluate the adaptive capacity and consequently the welfare of the animal. Animals are exposed to wide variety of environmental conditions during different seasons of the year. Seasons have marked effect on the production parameters as well as plasma hormones. Stressors of some systems are detectable as modifications of respiratory or heart rates, which are a valid index of social stress. Melanin pigment (eumelanin and pheomelanin) determines coat colour in mammals including cattle. Melanocortin 1 receptor (MC1R) gene is associated with pigmentation differences in mammals. Acquisition of a highly stable MC1R allele promotes black pigmentation, which helps in protection from UV damage. Another gene, PMEL, encodes a transmembrane protein called pre-melanosomal protein. PMEL is a melanocyte protein necessary for eumelanin deposition. Therefore, the above mentioned genes (MC1R and PMEL) divert the pathway of melanin synthesis towards eumelanin (true melanin) rather than pheomelanin. Coat characteristics are associated with heat tolerance and performance of animals. Skin color is also associated with the health condition of the individual. In animals, hair and skin pigmentation is a highly visible trait, however, to the best of our knowledge, little is known about the genetic variation responsible for large array of pigmentation.

*Author for correspondence
AIMS AND OBJECTIVES

To evaluate the changes in the level of expressions of skin color genes during summer and winter season.

MATERIAL AND METHODS

Ten Tharparkar heifers (2-3 years) were selected from the herd of National Dairy Research Institute (NDRI), Karnal, Haryana, India. The experiment was conducted during winter and summer season. These animals were maintained under normal conditions of feeding and management. From each animal, blood samples jugular vein in Lithium Heparin coated Vacutainer® tubes were collected (7.30 am) from at weekly interval for five times each during winter (Dec-Jan) and summer (May-June) seasons. The blood samples were centrifuged at 2300 rpm for 40 minutes to separate the plasma and buffy coat. The buffy coat obtained was used for lymphocyte separation to study the expression of the genes related to skin pigmentation i.e. MC1R and PMEL during both the seasons. Tyrosinase enzyme and hormonal parameters viz. Triiodothyronine (T₃) and Thyroxine (T₄) were studied in the plasma samples. The environmental variables in terms of minimum and maximum temperature, relative humidity were recorded and the calculated Temperature Humidity Index (THI) and data have been presented in Table 1.

Table 1: Environmental parameters recorded during winter and summer seasons

<table>
<thead>
<tr>
<th>Season</th>
<th>Temperature (°C)</th>
<th>Relative humidity (%)</th>
<th>THI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Maximum</td>
<td>Minimum</td>
<td>Dry bulb</td>
</tr>
<tr>
<td>Winter</td>
<td>17.2</td>
<td>4.1</td>
<td>14.9</td>
</tr>
<tr>
<td>Summer</td>
<td>36.4</td>
<td>18</td>
<td>37.6</td>
</tr>
</tbody>
</table>

Separation of lymphocytes

The buffy coat obtained was washed with 10 ml of 1:1 v/v Dulbeccos Phosphate Buffer Saline (DPBS; pH 4)). Total contents were carefully layered on lymphocyte separation medium at 3:1 v/v in sterile 15 ml polypropylene centrifuge tube and centrifuged at 2000 rpm for 40 min. at room temperature. The lymphocyte rich layer present between plasma and lymphocyte separation medium was collected in another sterile 15 ml polypropylene centrifuge tube containing 7 ml of DPBS and centrifuged at 1100 rpm for 10 min. The lymphocyte pellet collected at the bottom of polypropylene centrifuge tube was washed with 7 ml DPBS and centrifuged at 1100 rpm for 10 min., repeated twice.

RNA isolation

RNA was isolated from the harvested lymphocytes by RNeasy Mini Kit (Qiagen India Pvt. Ltd.) according to manufacturer’s protocol. RNA integrity was assessed in 1.5% agarose gel electrophoresis by observing rRNA bands corresponding to 28S and 18S. Possible genomic DNA contamination in RNA preparation was removed by using RNase-free DNase set (Qiagen India Pvt. Ltd.) according to manufacturer’s protocol. The absence of contaminant genomic DNA in RNA preparations was tested further using RNA as a template in real time PCR assays (RNA not reverse-transcribed to cDNA). Purity of RNA was checked in UV spectrometer with the ratio of the OD at λ260 and λ280 being >1.8.

cDNA synthesis

Total RNA from each sample was reverse transcribed into cDNA in a 20 μl reaction mixture using Revert Aid First strand cDNA synthesis kit (Fermentas, USA) by reverse transcription PCR according to the manufacturer’s protocol. Briefly, 200 ng RNA was reverse transcribed using Revert Aid M-MuLv reverse transcriptase (200 U/μl), RNase Inhibitor (20 U/μl), 10 mM dNTP mix (1 μl) oligo dT primers in 5X reaction buffer. The RT reaction was carried out at 25°C for 10 min, 42°C for 60 min, 75°C for 5 min in a thermal cycler (Bio-Rad, USA). The RT-PCR reaction was carried out in Applied Biosystems® 7500 Real-Time PCR systems using Maxima SYBR green qPCR master mix. Primers for MC1R, PMEL, GAPDH and β- actin were designed using primer 3.0 software (Table 2).
with the target genes two housekeeping [glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin] genes were amplified for relative expression measurements. Each sample had triple replicates and in all cases, samples of total RNA were used as negative control. Two housekeeping genes, GAPDH and β-actin were used in this study. The results indicated, GAPDH was better than β-actin. So expression data of GAPDH were used for analysis of relative expression data. Relative quantification of a target gene was done by comparing the expression levels of reference gene (GAPDH), as per the method⁶.

### Table 2: Primers sequence, annealing temperature and fragment size used for RT-PCR

<table>
<thead>
<tr>
<th>S/N</th>
<th>Primer</th>
<th>Sequence</th>
<th>Tₘ</th>
<th>Product size</th>
</tr>
</thead>
</table>
| 1   | MC1R   | F- ACAATGTCATCGACGTGCTC  
     |        | R- AGCTATGAAGGCAACCAAGGA | 58 | 245 |
| 2   | PMEL   | F- TTACTGACCAGGTGCCCTTC  
     |        | R- CTGTCACCAAAGTCCCAGGT | 59.5 | 169 |
| 3   | GAPDH  | F- CCAAGCTGTCTGTTGATCTGA  
     |        | R- AGCTTGACAAAGTGGTCGTTGAG | 55-60 | 218 |
| 4   | β-ACTIN | F- AGGCATCCTGACCCTCAAGTA  
     |        | R- GCTCGTTGTAGAAGGTGTGGT | 52-60 | 95 |

### Assay for plasma hormones

Tyrosinase level was determined using Bovine Tyrosinase ELISA kit (Cusabio Biotech Co., Ltd., Wuhan, China) according to the manufacturer’s instructions. The sensitivity of assay was <7.8 pg/ml. The intra-assay and inter-assay coefficients of variation were <8% and <10%, respectively.

Triiodothyronine (T₃) and Thyroxine (T₄) level was determined using Bovine RIA kit (Beckman Coulter Pvt. Ltd., New Delhi, India). The sensitivity of assay of T₃ was 0.5 ng/ml and the intra-assay and inter-assay coefficients of variation were 6.3% and 7.7%, respectively. The sensitivity of assay of T₄ was 16.7 ng/ml and the intra-assay and inter-assay coefficients of variation were 6.2% and 8.6%, respectively.

### Statistical analysis

Data of the present study were normally distributed as checked by Shapiro-Wilk test in SAS system. Data were analyzed by analysis of variance using SAS software, version (9.1) of the SAS system for Window, Copyright© (2011) SAS Institute Inc., Cary, NC, USA. Results were expressed as the means ± SEM. A difference with value P<0.05 was considered statistically significant. The correlation coefficient were carried out between the seasons using a correlation coefficient at the level of probability (P<0.01).

### RESULTS AND DISCUSSION

The mean values of ambient Temperature (Tₐ), Relative Humidity (RH) and Temperature - Humidity Index (THI) prevailed during the experimental period have been presented in Table 1. The mean THI value was found to be higher (>80) during summer than winter season. The results of the thyroid hormones (T₃ and T₄) have been presented in Table 3 and relative mRNA gene expression (MC1R and PMEL) in lymphocytes and plasma tyrosinase levels have been presented in Table 4.

The mean quantity of relative MC1R mRNA expression in the lymphocytes of Tharparkar heifers during winter and summer seasons were 11.52 ± 0.93 and 2.77 ± 0.26, respectively and the respective values of relative PMEL mRNA expression were 8.34 ± 0.60 and 4.41 ± 0.39 (Table 4). The relative MC1R and PMEL mRNA expression of Tharparkar heifers during the present investigation was found to be significantly (P<0.01) higher during winter than summer season. This may be probably due to the animals tried to expose more to sunlight during winter season. MC1R is one of the important genes associated with melanin pigmentation⁷. However, in addition to the MC1R genotype, regulation of MC1R expression also affects pigmentation⁸. UVR stimulates a retinal-dependent signaling cascade in human melanocytes that increases cellular melanin content⁹. Similarly¹⁰, reported that ultra-violet rays from
the sunlight stimulate the expression of melano- 
phore stimulating hormone receptors i.e. MC1R. 
MC1R has high binding affinity for a-MSH 11, 
which induce melanogenesis leading to darken- 
ing of the skin. The results of present study 
indicated that pigmentation was higher during 
winter than summer season in Tharparkar heifers. 
The relative PMEL mRNA expression 
in the lymphocytes of experimental animals during the 

present study showed similar trend with that of 
MCIR during the both seasons. Thus, 
the relative quantity of PMEL mRNA expression 
can be correlated with melanin pigmentation. 
Similar observed that PMEL is a melanocyte 
protein necessary for eumelanin deposition 
resulting in melanization. PMEL is also 
required to form functional amyloid fibrils 
during melanogenesis12.

Table 3 : Plasma triiodothyronine and thyroxine levels (ng/ml) in Tharparkar heifers during winter and summer season

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Triiodothyronine (ng/ml)</th>
<th>Thyroxine (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Winter</td>
<td>Summer</td>
</tr>
<tr>
<td>I</td>
<td>2.52 ± 0.15</td>
<td>1.83 ± 0.26</td>
</tr>
<tr>
<td>II</td>
<td>1.96 ± 0.10</td>
<td>1.98 ± 0.34</td>
</tr>
<tr>
<td>III</td>
<td>2.45 ± 0.19</td>
<td>1.53 ± 0.18</td>
</tr>
<tr>
<td>IV</td>
<td>2.57 ± 0.13</td>
<td>2.47 ± 0.31</td>
</tr>
<tr>
<td>V</td>
<td>2.73 ± 0.18</td>
<td>1.85 ± 0.25</td>
</tr>
<tr>
<td>Overall averages</td>
<td>2.45^a ± 0.08</td>
<td>1.93^b ± 0.13</td>
</tr>
</tbody>
</table>

The values are means ± S.E of ten observations on ten animals. The column with different superscripts differed significantly (P<0.05) between seasons and between breeds.

Table 4 : Plasma tyrosinase levels (pg/dl) and relative MC1R and PMEL mRNA expression in lymphocytes of Tharparkar heifers during summer and winter season

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Tyrosinase Winter</th>
<th>Summer</th>
<th>MC1R Winter</th>
<th>summer</th>
<th>PMEL Winter</th>
<th>Summer</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>48.52 ± 3.52</td>
<td>43.47 ± 3.38</td>
<td>7.79 ± 1.33</td>
<td>3.50 ± 0.62</td>
<td>5.27 ± 0.90</td>
<td>3.01 ± 0.41</td>
</tr>
<tr>
<td>II</td>
<td>51.99 ± 3.25</td>
<td>40.75 ± 3.02</td>
<td>18.99 ± 2.09</td>
<td>1.64 ± 0.12</td>
<td>10.61 ± 1.56</td>
<td>4.29 ± 0.68</td>
</tr>
<tr>
<td>III</td>
<td>44.94 ± 2.79</td>
<td>40.68 ± 3.07</td>
<td>11.21 ± 2.22</td>
<td>3.62 ± 0.73</td>
<td>8.79 ± 1.30</td>
<td>5.72± 1.02</td>
</tr>
<tr>
<td>IV</td>
<td>48.54 ± 3.03</td>
<td>40.89 ± 2.77</td>
<td>9.72 ± 1.37</td>
<td>2.09 ± 0.25</td>
<td>8.95 ± 1.20</td>
<td>3.57± 0.69</td>
</tr>
<tr>
<td>V</td>
<td>52.42 ± 3.72</td>
<td>39.85 ± 2.45</td>
<td>9.90 ± 1.51</td>
<td>2.97 ± 0.55</td>
<td>8.09 ± 1.29</td>
<td>5.46± 1.07</td>
</tr>
<tr>
<td>Overall Mean</td>
<td>49.28^a± 1.46</td>
<td>41.13^b± 1.33</td>
<td>11.52^a± 0.93</td>
<td>2.77^b± 0.26</td>
<td>8.34^c± 0.60</td>
<td>4.41^d± 0.39</td>
</tr>
</tbody>
</table>

The column with different superscripts differed significantly (P<0.05) between seasons and between breeds.

Animals adapted to hot environments have 
different skin colors and may be able to change 
their color over time to alter the absorption of 
solar radiation.13 The basis of coat colour in 
mammals including cattle is the presence or 
absence of melanin pigment (eumelanin and 
phaeomelanin). Eumelanin is responsible for 
black and brown colours and phaeomelanin for 
reddish brown, red tans and yellow.14 The higher 
relative quantity of MC1R mRNA expression 
in the lymphocytes of Tharparkar heifers during 
winter season reflects more availability of MSH- 
receptors (MC1R) to be bound with MSH. This 
is mainly due to MC1R has high binding affinity 
for α-MSH, the hormone responsible for 
pigmentation.14 Brunberg E. et al.,15 reported that PMEL causes 
the silver coat color in horse which is 
characterized by dilution of black pigment in 
hair.16 Also found that polymorphisms in this 
gene are associated with the dominant white, dun 
and smoky plumage color variants in chickens. 
Therefore, the results of the present study and 
literature available clearly indicate that MC1R 
and PMEL expression is associated with skin 
coat color. In the present study, the relative
expression of these genes changes with variation in environmental temperature. Eumelanin intensifies skin pigmentation and thus helps in photoprotection because of its efficiency in blocking ultraviolet rays (UV) and scavenging reactive oxygen species. The relative MC1R and PMEL mRNA expression showed significant (P < 0.01) negative correlation with THI. When the animals were less exposed to ultra – violet rays, due to fewer stimuli the relative MC1R mRNA expression was found to be reduced. However, the relative MC1R and PMEL mRNA expression showed significant (P<0.01) positive correlation with plasma tyrosinase, T3 and T4 levels in Tharparkar heifers. The results of the present study indicated that the relative MC1R and PMEL mRNA expression was higher during winter than summer. With the increase in the tyrosinase level, the relative MC1R and PMEL mRNA expression was also found to be higher. This suggested that MC1R and PMEL genes show positive relationship with tyrosinase enzyme. Therefore, the change in the level of MC1R and PMEL mRNA expression during winter and summer season is probably responsible for difference in pigmentation during the two seasons, since tyrosinase is the rate limiting enzyme for melanin biosynthetic pathway. The relative MC1R and PMEL mRNA gene expression changes with change in ambient temperature and metabolic hormones (T3 and T4). The expressions of these genes were found to be positively correlated with plasma T3 and T4 levels. Therefore, MC1R and PMEL gene must have some role in adaptability of animals to tropical climatic conditions. Reported the relation between relative MC1R mRNA expressions with skin color of animal. Thus, skin color of animal may play a role in regulating adaptive capacity of animals. 

**Thyroidal hormone (Triiodothyronine and Thyroxine)**

The mean values of Triiodothyronine (T3) and Thyroxine (T4) levels were 2.45 ± 0.08 and 55.45 ± 1.52 ng/ml respectively during winter season. Whereas the respective values of T3 and T4 were 1.93 ± 0.13 and 45.46± 1.38ng/ml (Table 3). The plasma T3 and T4 levels during summer were found to be significantly (P<0.05) higher during winter season than summer season. The result of the present study are in accordance to those of who reported cold environment as a stimulus to increase the thyrotropic hormone output thereby resulting in a higher concentration of thyroidal hormone in blood. The plasma thyroidal hormone showed negative correlation with THI. Lower (P<0.05) plasma concentration of T3 and T4 after 2 and 4 days exposure of animals to direct radiation was also reported. The thyroidal hormones showed positive correlation with tyrosinase enzyme and skin coat related genes (MC1R and PMEL).

**Tyrosinase enzyme**

The mean values of plasma tyrosinase enzyme during winter and summer seasons were 49.28 ± 1.46 and 41.13 ± 1.33pg/ml respectively (Table 4). The tyrosinase levels were found to be significantly higher during winter than summer season. This can be correlated with the increased melanogenesis during winter season being indicated by increased mean relative quantity of MC1R mRNA expression during winter season than summer season. The higher melanogenesis during winter may be associated with increased in plasma tyrosinase levels. Tyrosinase represents the critical regulatory point in the pathway of melanin formation. Similar findings have also been reported as tyrosinase is rate limiting enzyme for melanin synthesis. The plasma tyrosinase concentration showed significant (P<0.05) negative correlation with THI. However plasma tyrosinase concentration showed significant (P<0.05) positive correlation with expression of genes (MC1R and PMEL) and thyroidal hormones.

**CONCLUSION**

Under tropical climatic conditions with high levels of solar radiation, animals with a light coloured hair coat and darkly pigmented skin are believed to be better adapted. However, little is known about the genetic variation responsible for large array of pigmentation observed in animal population. There is a wide variation in the coat characteristics, including skin colour, between indigenous and crossbred cattle. The present study showed that genes responsible for skin pigmentation (MC1R and PMEL) were highly (P < 0.01) expressed during winter than summer season in Tharparkar heifers. The level of their expression altered with other heat stress related.
parameters. This indicates the importance of skin colour for adaptation of Tharparkar animals to tropical climatic conditions.

ACKNOWLEDGEMENT

The authors are thankful to the Director, NDRI, Karnal (India) for providing all necessary facilities carry out the research programme. The financial assistance for the research work received from National Initiative on Climate Resilient Agriculture (NICRA) project of Indian Council of Agricultural Research (ICAR), New Delhi, India.

REFERENCES

1. McRobie H. R. et al., Melanocortin 1 receptor (MC1R) gene sequence variation and melanin in the gray (Sciurus carolinensis), Fox (Sciurus niger) and red (Sciurus vulgaris) Squirrel, J. Hered., 105(3), 423-428, (2014).


