VARIATION IN GENE EXPRESSION OF LEPTIN AND INSULIN LIKE GROWTH FACTOR (IGF) GENES IN RESPONSE TO SEASONAL DIFFERENCES IN CAMEL

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Camels play an important socio-economic role within the pastoral and agricultural systems in the arid and semiarid zones of Asia and Africa. The one-humped dromedary (Camelus dromedaries), also called Arabian camel was domesticated in the Arabian Peninsula as a significant source of meat, milk and wool as well as a mean for transportation and sports for millions of people. Their bodies are very hardy and can resist a very fluctuating temperature from 34°C to 41.7°C and was the first mammalian genome to be sequenced in the Middle East (Soliman, 2015).

Leptin, is the hormone product of the obesity gene (LEP) which plays a major role in the regulation of body weight. This protein, which acts through the leptin receptor, functions as part of a signaling pathway that can inhibit food intake, has been found to change on seasonal manner. This gene gets its importance to discover valuable biomarker for recognizable proof of high performing animals with better adaptability and productivity (Qureshi et al., 2015).

Insulin-like Growth Factor-I (IGF-I) is a key stimulant of growth and development in animals. The production of IGF-I in the body is controlled by growth hormone (GH) and nutritional status. IGF-I has been hypothesized to be a possible biomarker which mediates the roles of physical activity and other factors on body composition and health outcomes (Nindl and Pierce, 2010).

Quantitative RT-PCR (RT-qPCR) is a fast method for accurate, sensitive and cost-effective changes in gene expression analysis as a robust and widely used methodology for biological investigation for very small amounts of specific nucleic acid sequences. Thus, it is essential to use reference genes such as GAPDH or β-actin which have been verified to be stably expressed within the specific experimental setting (Svingen et al., 2015).

Epigenetic modification could affect the expression of genes, and we triggered by environmental stimuli. They can persist throughout life or across multiple generations and can affect an individual's phenotype (Robinson et al., 2015). DNA
methylation is a well-characterized epigenetic modification that plays an important role in the regulation of gene expression (Ricceri et al., 2014). DNA methylation is coordinated with changes in the expression of stress-responsive genes to adapt to environmental changes (Kim et al., 2015).

The aim of this study was to assess the level of global DNA methylation of some economically-related genes (leptin and IGF) in association with thermal stress in camels.

MATERIALS AND METHODS

Sample Collection

Forty blood samples of Maghrabi female camels were kindly provided by the Camel Research Center, Desert Research Center (DRC), Marsa Matrouh. This city is subjected to Mediterranean coastal temperate climate in summer and cold winters prevail, where the highest temperature reaches 28°C in summer and less than 13°C in winter. Twenty samples were collected in the winter (w) (February, 2015) and twenty samples were collected in summer (s) (June, 2015) from the same animals as represented in Table (1).

RNA extraction and cDNA synthesis

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, GmbH, Germany) according to the manufacturer's protocol. RT-PCR was carried out using cDNA synthesis kit: GoScript™ Reverse Transcription System (Promega) according to the manufacturer's instructions. The PCR profile to generate cDNA started with one cycle at 25°C for 10 min. followed by 38 cycles at 37°C for 120 min. and 85°C for 5 min. Leptin, Insulin like Growth factor and β-actin primers were designed using Primer3 (v. 0.4.0) software and the sequences are shown in Table (2).

Quantitative Real Time PCR (qRT-PCR)

Real-time PCR reactions were carried out using the step one plus (Applied Biosystem machine, with 25 ng of cDNA, 500 nM of each primer, 10 µl of the SYBR green master mix (Quanti Tech SYBR Green kit, Qiagen, Gmbh Hilden, Germany) and RNase free water in a final volume of 20 µl. In the negative control, cDNA was replaced by RNase free water.

The program used for real-time PCR started with 15 min at 95°C, followed by 40 cycles of a denaturation step for 15 s at 95°C, an annealing step for 30 s at 58°C and an extension step for 30 s at 72°C; at the end of which the fluorescence was measured. Two replicates of real-time PCR reactions were performed for each sample.

Methylation analysis

Methylation level was performed according to The MethylFlash™ Methylated DNA Level Kit (EpiGenetek, USA). This kit employs the scientific basis of ELISA technique, and the results were read calorimetrically.
**Statistical analysis**

Data were organized in data sheet of excel and opened by IBM SPSS Statistics package-Version 20.0. The Kolmogorov-Smirnov test (KS test) was used as a nonparametric test of the equality of continuous, one-dimensional probability distributions to compare a sample with a reference probability distribution (one-sample KS test). Correlation coefficients were derived using Pearson’s correlation test. A \( p \)-value of is less than 0.05 was regarded as statistically significant.

**RESULTS AND DISCUSSION**

**Real time PCR analysis**

**Leptin Gene Expression in Different Seasons**

The levels of leptin gene expression were variable in the samples collected in winter season (Fig. 1 & Table 2), despite the obvious pattern of downregulation profile in comparison to control. The actual mechanism by which leptin and other milk production-related genes being regulated is hypomethylation cycle in response to heat stresses as reported by Berger *et al.* (2009). The profile obtained indicated that some samples were markedly affected (1w, 4w, 6w, 7w, 8w, 10w, 11w, 12w, 16w, 18w, 19w and 20w) compared to the rest of the sample (2w, 3w, 5w, 9w, 13w, 14w, 15w and 17w). Leptin gene expression was downregulated in all samples in comparison to control. Data analysis was performed using the SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). The simple t-test was used to analyze the difference between the data inside one sample, where the differences in gene expression were not significant \( (p = 0.343) \).

There was moderate correlation between leptin gene expression and methylation in winter season where the Pearson correlation value was equal to 0.498 which was significant \( (p = 0.025) \). Furthermore, the correlation between leptin gene expression and milk production in the winter season was weak where the Pearson correlation value \( (r) \) between leptin gene expression and milk production in winter season was 0.067 which was not significant \( (p = 0.786) \).

On the other hand, for the samples collected in the summer season (Fig. 2 & Table 2), the pattern of gene expression was much more variable. Leptin gene was upregulated in a number of samples (1S, 2S, 3S, 4S, 8S, 10S, 16S and 18S) and in others it was downregulated, there was a non-significant negative correlation between leptin gene expression and methylation in the summer season where the Pearson correlation value \( (r = -0.289) \) and \( (p = 0.216) \).

Moreover, the correlation between leptin gene expression and milk production in the summer season was negative and non-significant where the person correlation value was \( (r = -0.13) \) and \( (p = 0.571) \).

According to Mann-Whitney U Test (Fig. 3) there was significant differ-
ence \((p = 0.000)\) between the two samples groups (winter & summer) in terms of leptin gene expression, and this difference might be attributed to the atmospheric conditions at which the animals live.

**IGF Gene Expression in Different Seasons**

For IGF gene expression, the obtained profile in the winter season indicated a universal down-regulation in all the samples (Fig. 4 & Table 3). The obtained profile indicated that the IGF was affected greatly in some samples (1w, 6w, 7w, 8w, 10w, 11w, 16w, 18w, 19w and 20w) compared to the rest of the samples (2w, 3w, 4w, 5w, 9w, 12w, 13w, 14w, 15w and 17w). This profile could indicate the global hypomethylation pattern associated with reduced temperature in winter season, and this pattern was obtained in several previous works as mentioned by Del Vesco et al. (2015). SPSS (one simple-T test) was used to analyze the difference between the data inside one sample, where the differences in gene expression was clearly significant \(p\)-value \(< 0.05 \ (p = 0.000)\). This might also indicate that the individual variation between animals themselves was the cause behind these profiles (Bann et al., 2015).

There was a weak non-significant correlation \((r = 0.376) \ (p = 0.102)\) between IGF gene expression and methylation in winter season. Furthermore, the correlation between IGF gene expression and milk production in winter season was not significant as the Pearson correlation value was \((r = -0.059)\) and \((p = 0.8)\).

On the other hand, the obtained results (Fig. 5 & Table 3) showed that in the summer season IGF was downregulated in most samples while it exhibited up-regulation in some samples such as 1s, 4s and 13s in comparison to the control. SPSS showed that the differences in gene expression were clearly significant \((p = 0.02)\). The profile obtained indicated that IGF was down-regulated in some samples (5s, 19s and 20s) compared to others (2s, 3s, 6s, 9s, 16s and 17s) while it was upregulated in samples (1s, 4s and 13s).

There was a negative correlation between IGF gene expression and methylation in summer season where the Pearson correlation value was \((-0.067)\) and there was non-significant correlation between IGF gene expression and methylation in summer \((p = 0.780)\). The correlation between IGF gene expression and milk production in the summer season was negative and non-significant where the Pearson correlation value was equal to \(-0.28\) and \((p = 0.245)\).

Statistical analysis According to Mann-Whitney U Test, (Fig. 6) indicated that there was significant difference between the two sample groups (winter & summer) in IGF gene expression where \(p\)-value \(< 0.05 \ (p = 0.000)\), and this difference might be attributed to the atmospheric condition under which the animals live.

**Methylation level**

Methylation patterns were measured using MethylFlash™ DNA Quantification Kit (Colorimetric). For the samples
collected in the winter season, the obtained results indicated that the majority of samples were hypermethylated except for samples w7 and w8 (Fig. 7 & Table 4). Samples w17 and w18 showed the highest methylation levels with no remarkable change in gene expression.

Other samples also showed an increased methylation pattern which might substantiate the decrease of gene expression in the two genes under study (IGF and leptin). The obtained data were in partial agreement with those of Weyrich et al. (2015) who reported the same profile in different organisms.

Meanwhile, other samples (w4, w19 and w20) showed decreased levels of methylation compared to the rest of hypermethylated ones. These patterns were accordance with Jaenisch and Bird (2003) and with Varriale (2014) who studied the epigenetic variations as an indication on the level of gene expression in large animals exposed to variation of harsh environmental conditions.

Statistical analysis of the obtained data showed that there were significant differences in methylation levels ($p = 0.02$), Minor methylation levels were obtained for samples S1, S2, S12 and S16 which might reflect individual variation between animals as they were exposed primarily to the same climate atmosphere (Bossdorf et al., 2005; Novak and Mack, 2005; Ayroles et al., 2015). For samples S5, S6, S13 and S14 appreciable hypermethylation profile were obtained in Table (4).

Meanwhile, samples S4, S8, S9, S10, S11, S15, S18, S19 and S20 showed a hypermethylation pattern which might match the up-regulation of gene expression obtained in the genes under study (Fig. 8 & Table 4). The results are in agreement with those of Waterland et al. (2006), Gluckman et al. (2007), Sanchez et al. (2009), Bell et al. (2011), Martin et al. (2011), Richards (2011) and Houtkooper et al. (2012).

Statistical analysis of the obtained data showed that there were non-significant differences in methylation level ($p = 0.11$), According to Mann-Whitney U Test (Fig. 9) there was significant difference between the two sample groups (winter & summer) of methylation level where $p$-value = 0.020, and this difference might be attributed to the atmospheric condition at which the animal live.

**SUMMARY**

Epigenetic regulation of gene expression has proven to be a good biomarker for gene expression profiling. In the present study, Real-time PCR and Methylation level were performed to compare the levels of Leptin and IGF gene expression on 20 Maghrabi female camels exposed to variable temperatures (winter and summer). The results showed that hypermethylation prevailed in winter than in summer. A different profile was obtained in summer for both the two genes under study, as the hypomethylation was globally predominant.
It could be concluded that the seasonal variations and conditions of the external environment in which the animal lives affect the various proteins in gene expression for each of the two genes (leptin and insulin-like growth factor). Where there is an inverse relationship between gene expression and methylation level. This means that the drop of temperature in winter leads to an increase of the methylation level (hypermethylation); resulting in a decrease in gene expression (down-regulation). On the other hand, temperature was rising during the summer, leads to the decrease of methylation level (hypomethylation) resulting in an increase in gene expression (up-regulation) of the above-mentioned genes.

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Table (1): Productive data of 20 Magrabi female samples in winter and summer seasons.

| Ser. | Gender | Weight | Milk production |
|------|--------|--------|-----------------|
|      |        | Winter | Summer          |                 |
|      |        | Winter | Summer          |                 |
| 1    | female | 566    | 594             | 3000            |
| 2    | female | 655    | 587             | 4800            |
| 3    | female | 590    | 524             | 5000            |
| 4    | female | 485    | 529             | 5360            |
| 5    | female | 568    | 437             | 5150            |
| 6    | female | 550    | 463             | 4400            |
| 7    | female | 467    | 423             | 3800            |
| 8    | female | 538    | 418             | 5400            |
| 9    | female | 622    | 574             | -----           |
| 10   | female | 622    | 552             | 5000            |
| 11   | female | 513    | 442             | 5020            |
| 12   | female | 606    | 605             | 3800            |
| 13   | female | 621    | 527             | 5200            |
| 14   | female | 622    | 546             | 4200            |
| 15   | female | 636    | 614             | 6400            |
| 16   | female | 406    | 419             | 4380            |
| 17   | female | 532    | 501             | 4100            |
| 18   | female | 591    | 586             | 5000            |
| 19   | female | 608    | 602             | 4800            |
| 20   | female | 532    | 490             | 6400            |
Table (2): Primer Sequences for RT-PCR analysis of (Leptin, IGF) genes and β-actin as housekeeping gene.

| Gene        | F                        | R                        |
|-------------|--------------------------|--------------------------|
| Leptin      | 5’ GGA CCC CTC TGC CGA TTC 3’ | 5’ GCA CAG CTT CAA CAT AGG ACA GAT 3’ |
| IGF         | 5’ CCG TGA CCC ACG AAA TCT TC 3’ | 5’ CTGGGCTCTCGCCACAT3’ |
| β-actin     | 5’ GCA CCA CAC CTT CTA CAA TG 3’ | 5’ TGC TTGCTG ATC CAC ATCTG3’ |

Table (3): IGF & Leptin Genes Expression Analysis data of 20 Magrabi female samples collected in winter and summer seasons using Real Time PCR.

| IGF gene (winter) | IGF gene (summer) | Leptin gene (winter) | Leptin gene (summer) |
|-------------------|-------------------|----------------------|----------------------|
|                   |                   |                      |                      |
| Samples           | ΔΔCT              | Samples              | ΔΔCT              | Samples           | ΔΔCT              |
| Control           | 0.821             | Control              | 0.821             | Control           | 5.5330            | Control           | 5.5330            |
| W1                | -5.385            | S1                   | 0.730             | 1W                | -9.8790           | 1S                 | 2.8680            |
| W2                | -2.892            | S2                   | -0.570            | 2W                | -5.0860           | 2S                 | 0.7550            |
| W3                | -3.798            | S3                   | -0.181            | 3W                | -4.8090           | 3S                 | 1.5460            |
| W4                | -4.079            | S4                   | 0.479             | 4W                | -10.9910          | 4S                 | 2.7560            |
| W5                | -2.698            | S5                   | -8.829            | 5W                | -4.7090           | 5S                 | -7.6080           |
| W6                | -6.830            | S6                   | -0.459            | 6W                | -11.4920          | 6S                 | -0.1090           |
| W7                | -6.093            | S7                   | -1.620            | 7W                | -11.1070          | 7S                 | -2.0560           |
| W8                | -6.905            | S8                   | -2.162            | 8W                | -9.9690           | 8S                 | 2.6730            |
| W9                | -3.682            | S9                   | -0.800            | 9W                | -5.5070           | 9S                 | -2.2700           |
| W10               | -6.836            | S10                  | -1.278            | 10W               | -11.6960          | 10S                | 0.3810            |
| W11               | -5.506            | S11                  | -1.568            | 11W               | -11.5790          | 11S                | -1.1250           |
| W12               | -4.517            | S12                  | -1.326            | 12W               | -9.4980           | 12S                | -1.7990           |
| W13               | -3.176            | S13                  | 0.281             | 13W               | -7.6170           | 13S                | -0.3590           |
| W14               | -3.499            | S14                  | -1.977            | 14W               | -8.3640           | 14S                | -3.6690           |
| W15               | -2.312            | S15                  | -1.651            | 15W               | -3.7810           | 15S                | -1.5890           |
| W16               | -8.8393           | S16                  | -0.724            | 16W               | -10.7810          | 16S                | 1.7957            |
| W17               | -0.7055           | S17                  | -0.6341           | 17W               | -2.7085           | 17S                | 0.9272            |
| W18               | -7.4919           | S18                  | -1.814            | 18W               | -11.1026          | 18S                | -2.6292           |
| W19               | -6.9642           | S19                  | -2.5818           | 19W               | -10.7791          | 19S                | 0.4823            |
| W20               | -9.1072           | S20                  | -2.5062           | 20W               | -12.7563          | 20S                | -2.0603           |
Table (4): The concentration of 5-methylcytidin of 20 Magrabi female samples collected in winter and summer seasons

| No. of samples | Methylation | No. of samples | Methylation |
|----------------|-------------|----------------|-------------|
| C-             | 0.5151      | C-             | 0.5151      |
| C+             | 9.3666      | C+             | 9.3666      |
| S1             | 0.5151      | W1             | 5.8260      |
| S2             | 0.5151      | W2             | 16.4478     |
| S3             | 2.2854      | W3             | 11.1369     |
| S4             | -3.0255     | W4             | 2.2854      |
| S5             | 12.9072     | W5             | 4.0557      |
| S6             | 30.6102     | W6             | 4.0557      |
| S7             | 4.0557      | W7             | -8.3364     |
| S8             | -1.2552     | W8             | -3.0255     |
| S9             | -1.2552     | W9             | 9.3666      |
| S10            | -1.2552     | W10            | 4.0557      |
| S11            | -3.0255     | W11            | 5.8260      |
| S12            | 0.5151      | W12            | 4.0557      |
| S13            | 9.3666      | W13            | 4.0557      |
| S14            | 18.2181     | W14            | 9.3666      |
| S15            | -1.2552     | W15            | 5.8260      |
| S16            | 0.5151      | W16            | 7.5963      |
| S17            | 4.0557      | W17            | 28.8399     |
| S18            | -3.0255     | W18            | 25.2993     |
| S19            | -6.5661     | W19            | 2.2854      |
| S20            | -1.2552     | W20            | 0.5151      |
Fig. (1): Leptin gene expression analysis of 20 Maghrabi camel samples (1W to 20W) collected in winter using real time PCR.

Fig. (2): Leptin gene expression analysis of 20 Maghrabi camel samples (1S to 20S) collected in summer using real time PCR.

Fig. (3): The distribution of Leptin gene results across categories of weather for all samples.
Fig. (4): IGF gene expression analysis of 20 Maghrabi camel samples (1W to 20W) collected in winter season using real time PCR.

Fig. (5): IGF gene expression analysis of 20 Maghrabi camel samples (1S to 20S) collected in summer season using real time PCR.

Fig. (6): The distribution of IGF gene results across categories of weather for all samples.
Fig. (7): The methylation level of 20 Maghrabi camel samples (1W to 20W) collected in winter season.

Fig. (8): The methylation level of 20 Maghrabi camel samples (1W to 20W) collected in summer season.

Fig. (9): The distribution of 5-Methylcytidin across categories of weather for all samples.
