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

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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 down-regulation profile in comparison to control. The actual mechanism by which leptin and other milk production-related genes being regulated is hyper-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 was (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 upregulation 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.

REFERENCES

Ayroles, J. F., S. M. Buchanand, C. O. Learya, K. S. Kakariaa, J. K. Grenierc, A. G. Clarkc, D. L. Hartla and B. L. de Bivort (2015). Behavioural idiosyncrasy reveals genetic control of phenotypic variability. PNAS, 112: 6706-6711.

Bann, D., J. M. P. Holly, H. Lashen, R. Hardy, J. Adams, D. Kuh, K. K. Ong and Y. Ben-Shlomo (2015). Changes in Insulin-Like Growth Factor-I and -II Associated with Fat but not Lean Mass in Early Old Age Obesity, 23: 692-698.

Bell, J. T., A. A. Pai1, J. Pickrell, D. J. Gaffney, R. P. Regi, J. F. Degner, Y. Gilad and J. K. Pritchard (2011). DNA methylation patterns associate with genetic and gene expression variation in HapMap cell lines. Genome Biology, 10: 1-13.

Berger, S. L., T. Kouzarides, R. Shiekhattar and A. Shilatifard. (2009). An operational definition of epigenetics. Genes Dev., 23: 781-783.

Bossdorf, O., H. Auge, L. Lafuma, W. E. Rogers, E. Siemann and D. Prati (2005). Phenotypic and genetic differentiation between native and introduced plant populations. Oecologia, 144: 1-11.

Del Vesco, A. P., E. Gasparino, D. O. Grieser, V. Zancanela, D. M. Voltolini, A. S. Khatlab, S. E. F. Guimarães, M. A. M. Soares and A. R. O. Neto (2015). Effects of methionine supplementation on the expression of protein deposition-related genes in acute heat stress-exposed broilers. PLoS ONE, 10: e0115821. doi:10.1371.

Gluckman, P. D., K. A. Lillycrop, M. H. Vickers, A. B. Pleasants, E. S. Phillips, A. S. Beedle, G. C. Burdge and M. A. Hanson (2007). Metabolic plasticity during mammalian development is directionally dependent on early nutritional status. Proc. Natl. Acad. Sci. USA, 104: 12796-12800.

Houtkooper, R. H., E. Pirinen and J. Auwerx (2012). Sirtuins as regulators of metabolism and healthspan.

Nat. Rev. Mol. Cell Biol., 13: 225-238.

Jaenisch, R. and A. Bird (2003). Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nature Genetics, 33: 245-254.

Kim, J. M., T. Sasaki, M. Ueda1, K. Sako and M. Seki (2015). Chromatin changes in response to drought, salinity, heat, and cold stresses in plants. Front Plant-Science, 6:114. doi: 10.3389/ fpls.2015.00114.

Martin, L. B., A. L. Liebl, J. H. Trotter, C. L. Richards, K. McCoy and M. W. McCoy (2011). Integrator networks: illuminating the black box linking genotype and phenotype. Int. Comp. Biol. doi: 10.1093/icb/ icr049.

Nindl, B. C. and J. R. Pierce (2010). Insulin-like growth factor I as a biomarker of health, fitness, and training status. Med. Sci. Sports Exerc., 42: 39-49.

Novak, S. J. and R. N. Mack (2005). Genetic bottlenecks in alien plant species: influences of mating systems and introduction dynamics. In: Species Invasions: Insights into Ecology, Evolution, and Biogeography (eds Sax D. F., Stachowicz J. J., Gaines S. D.), 201-228.

Qureshi, Z. I., A. H. Farid, M. E. Babar and T. Hussain (2015). Leptin gene polymorphism in lohi, kajli and spili breeds of sheep. Pak. Vet. J., 35: 321-324.

Ricceri, F., M. Trevisan, V. Fiano, C. Grasso, F. Fasanelli, Ch. L. Scoccianti, D. A. Marco, G. Tos, P. Vineis and C. Sacerdote (2014). Seasonality modifies methylation profiles in healthy people. PLOS ONE, Vol. 9, e106846.

Richards, E. J. (2011). Natural epigenetic variation in plant species: a view from the field. Curr. Opin. Plant Biol., 14: 204-209.

Robinson, N., H. Moghadam and T. Mørkøre (2015). Epigenetics-potential for programming fish for aquaculture? J. Mar. Sci. Eng., 3: 175-192.

Sanchez, C., A. Oskowitz and R. R. Pochampally (2009). Epigenetic reprogramming of IGF1 and leptin genes by serum deprivation in multipotential mesenchymal stromal cells. Stem Cells, 27: 375-382.

Soliman, M. K. (2015). Functional anatomical adaptations of dromedary (*Camelus Dromedaries*) and ecological evolutionary impacts in KSA. Inter. Conf. on Plant, Marine and Environmental Sciences Jan. 1-2, 2015 Kuala Lumpur (Malaysia).

Svingen, T., H. Letting, N. Hadrup, U. Hass and A. M. Vinggaard (2015). Selection of reference genes for

quantitative RT-PCR (RT-qPCR) analysis of rat tissues under physiological and toxicological conditions. PeerJ 3:e855, DOI 10.7717/peerj.855

Varriale, A. (2014). DNA Methylation, Epigenetics, and Evolution in Vertebrates: Facts and Challenges. International Journal of Evolutionary Biology, http:// dx.doi.org/10.1155/ 2014/475981.

Waterland, R. A., J. R. Lin, C. A. Smith and R. L. Jirtle (2006). Post-weaning diet affects genomic imprinting at the insulin-like growth factor 2 (Igf2) locus. Hum. Mol. Genet., 15: 705-716.

Weyrich, A., D. Lenz, M. Jeschek, T. H. Chung, K. Rubensam, F. Goritz, K. Jewgenow and J. Fickel (2015). Paternal intergenerational epigenetic response to heat exposure in male Wild guinea pigs. Mol. Ecol. doi:10.1111/ mec.13494.

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	3400
2	female	655	587	4800	4600
3	female	590	524	5000	4400
4	female	485	529	5360	4600
5	female	568	437	5150	4800
6	female	550	463	4400	4100
7	female	467	423	3800	3100
8	female	538	418	5400	4800
9	female	622	574	-----	-----
10	female	622	552	5000	4600
11	female	513	442	5020	4600
12	female	606	605	3800	3100
13	female	621	527	5200	4880
14	female	622	546	4200	3700
15	female	636	614	6400	5800
16	female	406	419	4380	3600
17	female	532	501	4100	3800
18	female	591	586	5000	4600
19	female	608	602	4800	4200
20	female	532	490	6400	5650

Table (2): Primer Sequences for RT-PCR analysis of (Leptin, IGF) genes and β-actin as housekeeping gene.

Leptin	F	5' GGA CCC CTC TGC CGA TTC 3'
	R	5' GCA CAG CTT CAA CAT AGG ACA GAT 3'
IGF	F	5' CCG TGA CCC ACG AAA TCT TC3'
	R	5'CTTGGGCTCCTGCCACAT3'
β-actin	F	5' GCA CCA CAC CTT CTA CAA TG 3'
	R	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	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	16 W	-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

The concentration of 5-methylcytidin (summer)		The concentration of 5-methylcytidin (winter)	
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

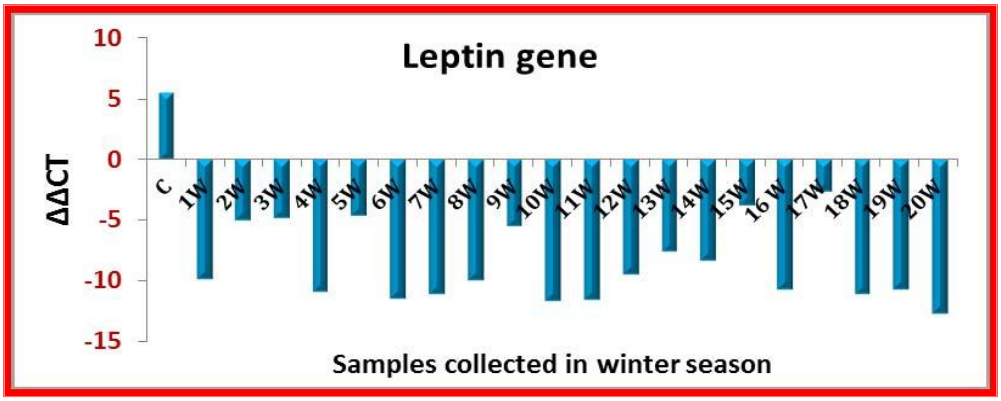

Fig. (1): Leptin gene expression analysis of 20 Maghrabi camel samples (1W to 20W) collected in winter using real time PCR.

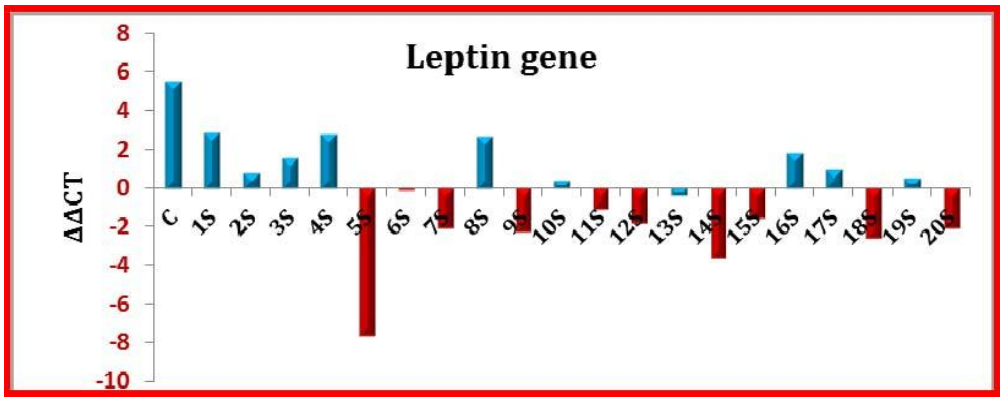

Fig. (2): Leptin gene expression analysis of 20 Maghrabi camel samples (1S to 20S) collected in summer using real time PCR.

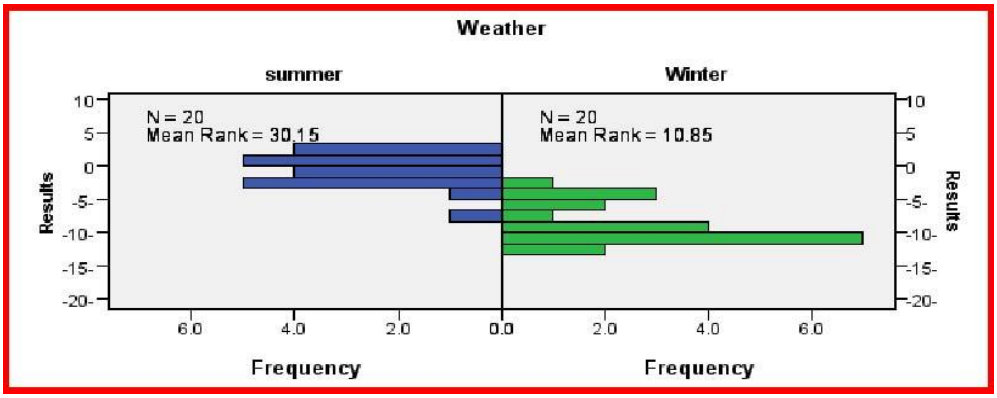

Fig. (3): The distribution of Leptin gene results across categories of weather for all samples.

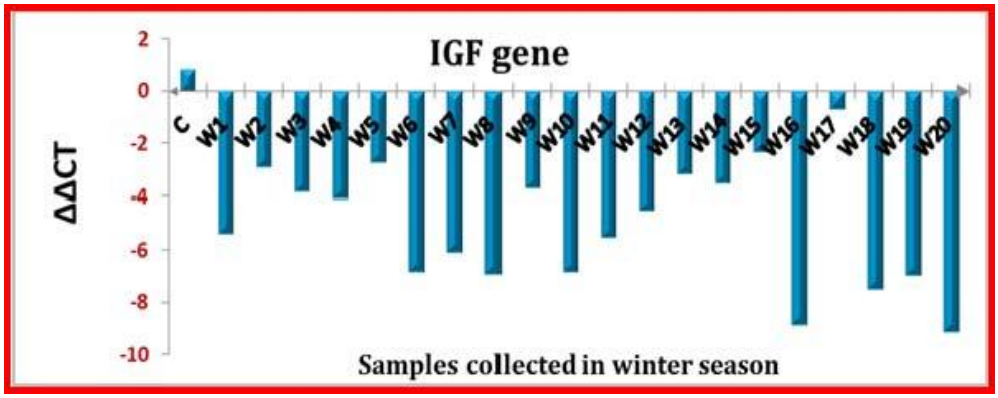

Fig. (4): IGF gene expression analysis of 20 Maghrabi camel samples (1W to 20W) collected in winter season using real time PCR.

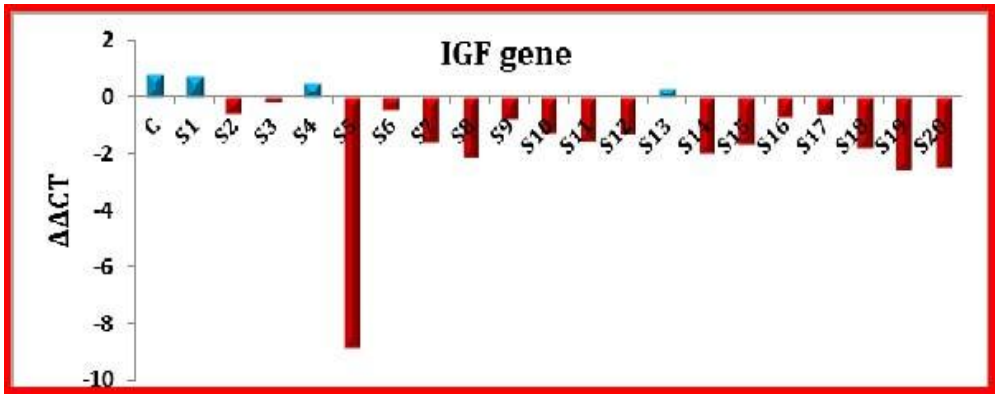

Fig. (5): IGF gene expression analysis of 20 Maghrabi camel samples (1S to 20S) collected in summer season using real time PCR.

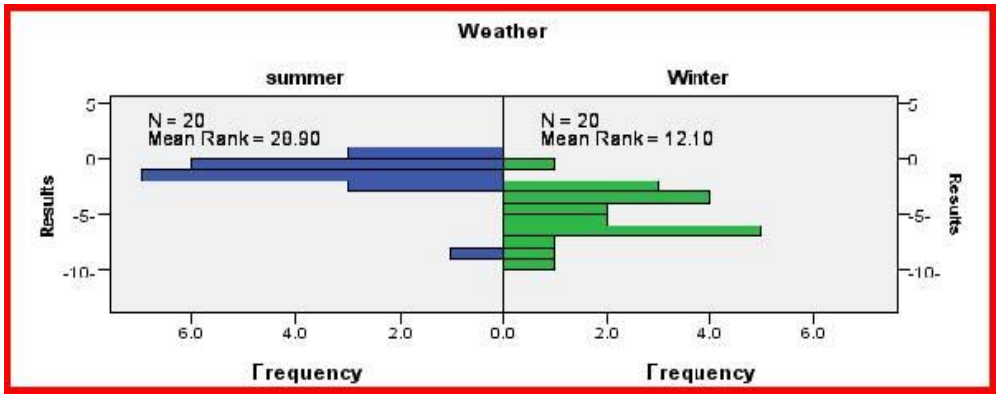

Fig. (6): The distribution of IGF gene results across categories of weather for all samples.

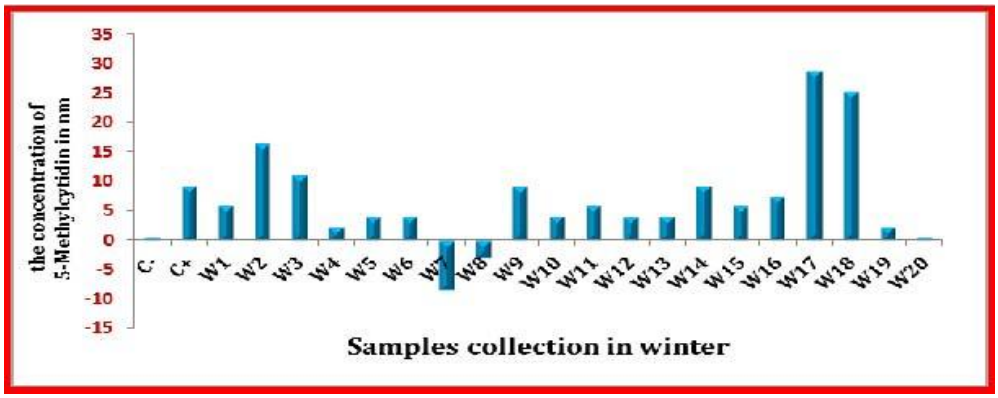

Fig. (7): The methylation level of 20 Maghrabi camel samples (1W to 20W) collected in winter season.

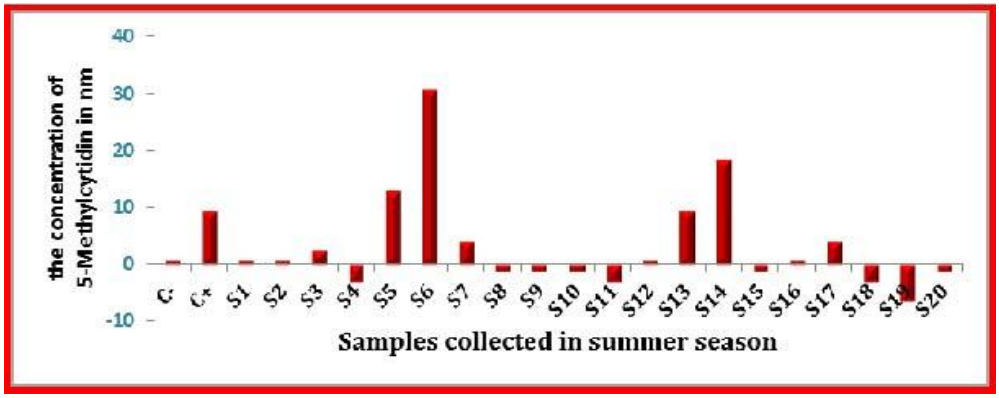

Fig. (8): The methylation level of 20 Maghrabi camel samples (1W to 20W) collected in summer season.

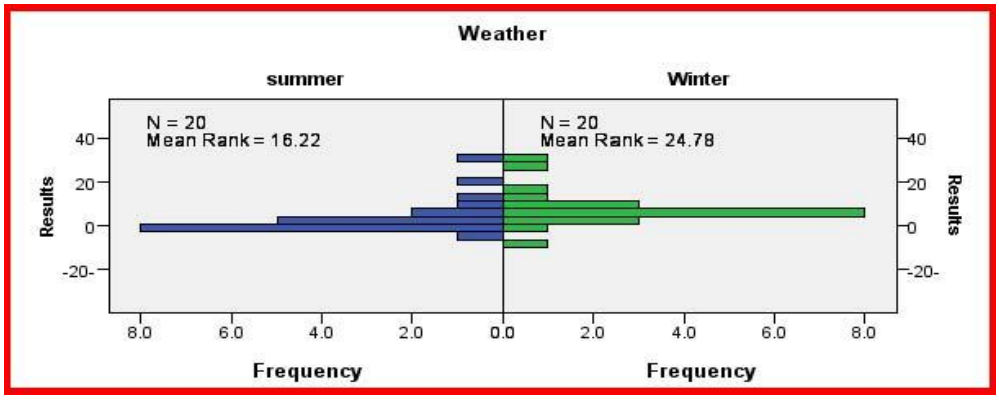

Fig. (9): The distribution of 5-Methylcytidin across categories of weather for all samples.