Possible implication of melatonin receptor 1A and Arylalkylamine N acetyltransferase genes polymorphisms for seasonal reproduction in Egyptian sheep breeds

(Received: 02. 05. 2018; Accepted: 20.05.2018)

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ABSTRACT

This study was carried out for detection of polymorphisms in melatonin receptor 1A (MTNR1A) and Arylalkylamine N acetyltransferase (AA-NAT) genes and their association with reproductive traits. Blood samples of 126 Animal from three Egyptian sheep breeds were collected. DNA was extracted and subjected to PCR-RFLP using RsaI and SmaI enzymes. Two alleles (C and T), three genotypes (CC, CT and TT) for MTNR1A gene also (A and G), (GG, GA, and AA) for AA-NAT gene were detected. Alleles C and A, genotypes CT and GA showed the highest frequency for MTNR1A and AA-NAT genes respectively. Association analysis of MTNR1A SNP revealed a significant association in Ossimi and Rhmani breeds with age of first lambing and C allele seems to be the favorable allele. Results for AA-NAT SNP demonstrate significant differences in Ossimi with age of first lambing and litter size and in Rhmani with lambing interval and G allele seems to be the desirable allele. Concerning to first conception season the study revealed that ewes carrying CT exhibited significantly lower age of first lambing in unfavorable season. Also, GG ewes exhibited significantly lower age of first lambing in early favorable followed by unfavorable seasons. According to personal knowledge this is the first study concerned with this association in Egyptian sheep breed. In conclusion: the polymorphisms achieved in this study could be measured as genetic markers suitable for improving reproductive efficiency during unfavorable season and the obtained desirable genotypes could be taken into account in new genetic selective schemes.

Keywords: *MTNR1A, AA-NAT, Egyptian sheep, polymorphism, Reproductive seasonality, PCR-RFLP.*

INTRODUCTION

The use of molecular genetics technologies potentially offers a way to select breeding animal at an early age and can be used to select a wide range of traits and can enhance reliability in predicting the mature phenotype of the individual (Naqvi 2007). One approach is known as Marker assisted selection (MAS) which may represent a possible option for designing a suitable breeding scheme for livestock with productive and reproductive traits. In Egypt, sheep contribute about 6% of the total red meat production. Rahmani, Ossimi, and Barki, are of the main sheep breeds (Galal *et al.,* 2005).

Unlike most domestic livestock species sheep reproduction is widely known with a marked seasonality of breeding activity (Rosa and Bryant, 2003). Teyssier *et al*. (2011) stated that changes in day length may act as a major factor controlling seasonal changes in oestrous activity in sheep breeds with maximal reproductive activity associated with short days and indicated a possibility for selection within these breeds for more continuous oestrous activity. Melatonin is called the "hormone of darkness,", As its production is controlled by day/night alteration (Rosa and Bryant, 2003). Short photoperiods influence positively on melatonin level. The increasing melatonin levels will stimulate the pituitary gland to release follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Falk, 2013). Melatonin is linked with two specific high affinity receptors,1A and 1B, however Melatonin receptor 1A (MTNR1A) is the main receptor which mediate the reproductive and circadian actions of melatonin in sheep. Polymorphism of MTNR1A gene has been documented to be associated with seasonal reproduction in sheep breed (Carcangiu *et al.,*2009) [a] and buffalo (Barbosa *et al*., 2017). Arylalkylamine N-acetyltransferase (AA-NAT) is called "the Timezyme" as it play a unique role in vertebrate time keeping (Klein, 2007) and consider the rate-limiting enzyme in Melatonin (MLT) biosynthesis therefore, any polymorphism in the gene of AA-NAT, may contribute to the variability of melatonin production and influence seasonally estrus response in the sheep population (Koike *et al*.,2013 and Oner *et al*., 2014). As, the determination of the genetic diversity of indigenous sheep in Egypt and its association with reproductive traits has not been sufficiently studied, the present investigation was aimed to fulfill towards the identification of the polymorphisms at the melatonin receptor gene (MTNR1A) and Aryl alkyl amine N-acetyl transferase (AA-NAT) gene in three Egyptian sheep breeds and their relationship with some reproductive performance traits which might be used as genetic marker to improve out of season fertility.

MATERIALS AND METHODS

Animals and data records

The present study was conducted on a total of 126 animals belonging to three Egyptian sheep breeds Ossimi n=66, Rahmani n=41, and Barki n=19 (raised in Animal Production Research Station, Sakha, Kafrelshiekh. Age at First Lambing (AFL), lambing Interval (LI), litter size (LS) and fertility rate were used for evaluating the reproductive performance. The seasons of conception was defined as early favorable (EF; Sep to Nov), late favorable (LF; Dec to Feb), or unfavorable (UF; Mar to Aug).

Ethical approval

The animal experiment was conducted after approval of Institutional Animal Care and Use committee, Cairo University (CU-IACUC) for the purpose of control and supervision of experiments on animals with minimal stress with approval number CUIIS5117.

Blood sampling and genomic DNA extraction

Blood samples were collected by jugular vein puncture into EDTA vacuum tubes and kept in -20°C until using. Genomic DNA was extracted using a genomic DNA extraction purification kit (Quick-g DNA MiniPrep™– Zymoresearch, USA). DNA quantity and purity for each sample were assessed by spectrophotometer and agarose gel electrophoresis, which were suitable for a PCR protocol application.

Polymerase chain reaction for *MTNR1A* gene and AA-NAT gene

PCR fragment of 824 bp the main part of the exon II of the ovine MTNR1A gene of the sequence (GeneBank U14109) was amplified with specific primers as described by Messer *et al.* (1997) with sequence of forward: 5'-TGT GTT TGT GGT GAG CCT GG-3' and reverse: 5'-ATG GAG AGG GTT TGC GTT TA-3'. While the PCR fragment of 1142 bp of AA-NAT gene (GeneBank JX444551.1) including the sequences of concluded part of exon1 (152 bp), intron 1 (290 bp), whole exon 2 (155 bp), intron 2 (338 bp), part of exon 3 (207 bp). The sequence was amplified with specific primers as described by Ding-ping *et al.* (2012) with sequence of forward: 5'-AGC GTC CAC TGC CTG AAA C-3' and reverse: 5'- GGG ATG GAA GCC AAA CCT C-3' (Invitrogen by Thermofisherscientific, EU). The amplifications were applied on Mastercycler nexus gradient thermocycler (Eppendorf AG 2231 Hamburg ,Germany) in a total volume of 25µl containing 1µl of Tempelate DNA, 1x Dreamtaq^{TM} Green master mix (Thermofisherscientific ,EU), 10 pmol/µl of each primer and Nuclease free water with addition of 1 µl Bovine serum albumin (2.5mg/ml) with following temperatures profile consisting of an initial denaturation at 94 ℃ for 5 min, followed by at 35 cycle program with denaturation at 95 ℃ for 1 min, annealing at 62 ℃ for 1 min, elongation at 72 ℃ for 1 min and final elongation at 72 ℃ for 10 min for MTNR1A gene, initial denaturation at 94 ℃ for 5 min, followed by at 35 cycle program with denaturation at 95 ℃ for 45sec, annealing at 60 ℃ for 45sec, elongation at 72 ℃ for 2 min and final elongate ion at 72 ℃ for 10 min for AA-NAT gene. The PCR products were detected on 1.5 % ethidium bromide stained agarose gel electrophoresis.

Restriction fragment length polymorphism (RFLP)

The PCR product of MTNR1A gene was digested by RsaI restriction enzyme (Thermo scientific FastDigest,EU) according to Saxena *et al.* (2015) . The reaction conducted in 30 µl final volume; containing 10µl of amplicon ,1 µl of enzyme, 2µl of 10x fast digest buffer and 17 µl nuclease free water) at 37^{o}c for 90 min followed by deactivation process at 65^{o}c for 20 min. In the same sequence PCR product of the AA-NAT gene was digested by SmaI restriction enzyme (New England Biolabs cutsmart^{TM}, MA, USA) according to Ding-Ping *et al.* (2012). The reaction was conducted in 40 µl reaction volume containing 16 µl of amplicon, 1µl of enzyme, 5µl of 10X NE Buffer and 18 µl of nuclease free water at 25^{o}c for one 90 min followed by deactivation at 65^{o}c for 20 min, the results of digestion were visualized by 3% agarose gel electrophoresis stained with ethidium bromide.

Statistical Analysis

Estimates of genotypic and allelic frequencies, heterozygosity, number of effective alleles and Hardy-Weinberg Equilibrium test for each population breed were carried out using GENE POP software, version (4.2) according to Yeh *et al.* (1999).To test the association of different conformational patterns with the reproductive traits, the preliminarily analysis of data were subjected to two -way analysis of variance with (Breed, Conformation patterns, First Conception Season, Season of Lambing) as fixed effects using General Linear Model (GLM) procedure of the Statistical Analysis System (SAS 2002) program, version (9.1). The following linear model for reproductive traits studied is used: -

$Y_{ijklmn} = \mu + B_i + G_j + F_k + S_l + P_m + (GF)_{jk} + (GS)_{jl} + e_{ijklmn}$

Where:

Yijklmn : The measurements of reproductive traits, μ : Overall mean, Bi: The fixed effect of the i^{th} breed (1, 2, 3; i.e. 1=Ossimi, 2=Rhmani, 3=Barki), Gj :The fixed effect of the j^{th} conformation pattern (1, 2, 3), Fk : The fixed effect of the k^{th} First Conception Season (1, 2, 3; i.e. 1=LF, 2=UF, 3=EF), Sl : The fixed effect of the Season of Lambing (1, 2, 3; i.e. 1= LF, 2= UF, 3= EF), Pm: The fixed effect of the ^{ith}Parity (1, 2 … 13), (GF) jk : The fixed effect of the j^{th} conformation pattern nested within the ^{kth}First Conception Season.(GS), jl : The fixed effect of the jth conformation pattern nested within the ^{lth}Season of Lambing, eijklmn : Random residual errors assumed to be normally distributed with mean = 0 and variance=σ2e .

RESULTS

PCR amplification of MTNR1A and AA-NAT gene fragment

Exon II region of MTNR1A gene was successfully amplified using specific primer giving rise to an amplicon of 824 bp as shown in Figure (1A). The AA-NAT gene was successfully amplified using specific primer giving rise to an amplicon of 1142 bp as shown in Figure (1B).

(1A) **(1B)**

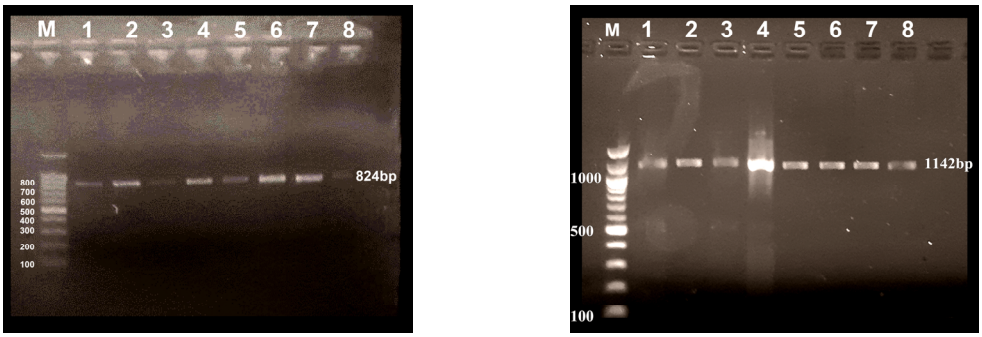

Fig. (1A): Agarose gel electrophoresis (1.5 %) showing PCR products of exon II MTNR1A gene. All lanes showing a single expected specific band of 824 bp. Fig. (1B): Agarose gel electrophoresis (1.5 %) showing PCR products of AA-NAT gene (part of exon I 152 bp, intron I 290 bp, whole exon II 155 bp, intron II 338 bp, part of exon III 207 bp). All lanes showing expected specific amplified band of 1142bp. M represent the 100bp DNA ladder.

RFLP and genotyping of exon II MTNR1A using RsaI enzyme

The result for RsaI enzyme revealed 3 genotypes CC (411 bp, 267 bp); CT (411,290.267) and TT (411 bp, 290 bp) (Figure 2). The three genotypes were identified in Ossimi and Rhmani breeds while only two genotypes (CC and CT) was detected in Barki breed with absence for TT genotype.

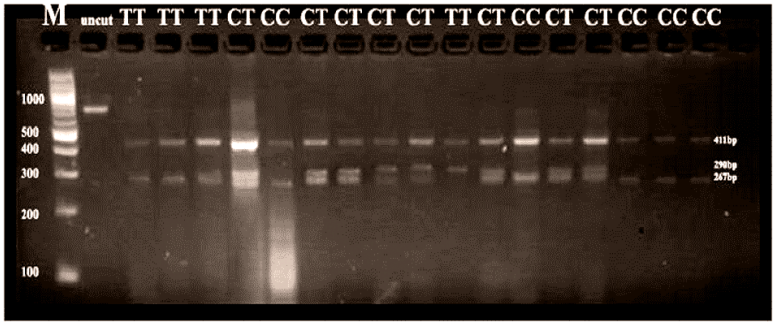

Fig. (2): Agarose gel electrophoresis (3%) showing PCR-RFLP of exon-II of MTNR1A using RsaI enzyme. M represent the 100bp DNA ladder, second lane represent uncut 824bp. Lanes represent genotype CC (411bp, 267 bp) and TT (411 bp, 290 bp) and CT (411, 290, 267bp).

Table (1): Frequency of genotypes and alleles for MTNR1A gene locus for different Egyptian sheep breeds on base of RFLP.

Breeds	Observed Genotype frequencies			Expected Genotype frequencies			Allele frequencies		UHe	Hardy-Weinberg Equilibrium	
	CC	CT	TT	CC	CT	TT	C	T		X^2-test	*P* value
Ossimi N=66	0.409 (27)	0.455 (30)	0.136 (9)	0.405	0.463	0.132	0.636	0.364	0.466	0.021	0.885^{ns}
Rhmani N=41	0.260 (11)	0.439 (18)	0.293 (12)	0.238	0.500	0.262	0.488	0.512	0.506	0.605	0.437^{ns}
Barki N=19	0.632 (12)	0.368 (7)	0 (0)	0.666	0.301	0.034	0.816	0.184	0.309	0.969	0.325^{ns}
Total N=126	0.397 (50)	0.437 (55)	0.167 (21)	0.378	0.474	0.148	0.615	0.385	0.427	0.770	0.380^{ns}

Where: UHe= Unbiased Expected Heterozygosity, Number between brackets= No. of observed genotype.

Table (2): Frequency of genotypes and alleles for AA-NAT gene locus for different Egyptian sheep breeds on base of RFLP.

Breeds	Observed Genotype frequencies			Expected Genotype frequencies			Allele frequencies		UHe	Hardy-Weinberg Equilibrium	
	GG	GA	AA	GG	GA	AA	G	A		X^2-test	*P* value
Ossimi N=66	0.090 (6)	0.333 (22)	0.576 (38)	0.066	0.382	0.551	0.258	0.742	0.385	1.089	0.297^{ns}
Rhmani N=41	0.244 (10)	0.585 (24)	0.171 (7)	0.288	0.497	0.215	0.537	0.463	0.503	1.285	0.257^{ns}
Barki N=19	0 (0)	0.842 (16)	0.158 (3)	0.177	0.488	0.335	0.421	0.579	0.501	10.050	0.002^{**}
Total N=126	0.127 (16)	0.492 (62)	0.381 (48)	0.139	0.468	0.393	0.373	0.627	0.463	0.340	0.560^{ns}

Where: UHe = Unbiased Expected Heterozygosity, Number between brackets= No. of observed genotype.

Genotyping and allelic frequency for MTNR1A receptor gene

The allelic and genotypic frequencies of MTNR1A SNP 606C>T are shown in Table (1). The allelic frequency for Ossimi, Rhmani and Barki breeds was 0.636, 0.488 and 0.816 respectively for allele C; 0.364, 0.512 and 0.184 respectively for allele T. Also, genotype frequency for Ossimi, Rhmani and Barki breeds was 0.409, 0.260 and 0.632 respectively for genotype CC; 0.455, 0.439 and 0.368 respectively for genotype CT and 0.136, 0.293 and 0.0 respectively for genotype TT. On the whole, the C allele showed high frequency (0.615) and heterozygous genotype CT has higher frequency (0.437). The observed and expected genotype frequencies were approximately the same. Results of the genetic diversity estimated by unbiased heterozygosity (UHe) demonstrate highest degree in Rhmani (0.506) followed by Ossimi (0.466) and Barki (0.309). The obtained exact P values for X^2 test in all populations confirm the accordance with the Hardy-Weinberg distribution in all investigated breeds.

RFLP and genotyping of exon III AA-NAT gene using SmaI enzyme

The result for SmaI enzyme reveal 3 genotypes AA (516bp, 371bp, 255bp); GG (371bp, 333bp, 255bp, 183bp) and GA (516bp,371bp, 333bp, 255bp, 183bp) (Fig. 3). All the three genotypes were identified in Ossimi and Rhmani breeds while in Barki breed no GA genotype was detected.

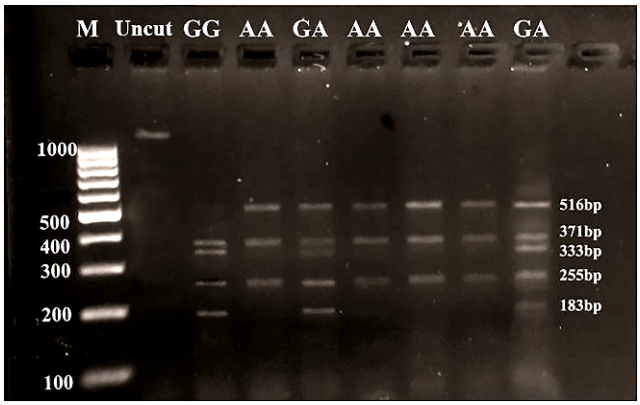

Fig. (4): Agarose gel electrophoresis (3%) showing PCR-RFLP. M represent the 100bp DNA ladder, second lane represent uncut 1142bp. Lanes represent genotype GA (516bp, 371bp, 333bp, 255bp, 183bp) and GG (371bp, 333bp, 255bp, 183bp) and AA (516bp, 371bp, 255bp).

Table (3): Effect of SNP of MTNR1A gene on reproductive traits in Egyptian sheep breeds.

Breeds	Genotype	Age at First Lambing (AFL)				Lambing Interval (LI)			Litter size (LS)			Fertility		
		N	LSM	±	SE	LSM	±	SE	LSM	±	SE	LSM	±	SE
Ossimi	CC	27	1006.60^{ab}	±	33.25	453.30	±	39.09	1.01	±	0.03	0.58	±	0.08
	CT	30	953.72^{a}	±	36.83	493.60	±	5.47	1.07	±	0.04	0.59	±	0.08
	TT	9	1176.55^{b}	±	56.59	447.20	±	78.75	1.00	±	0.07	0.57	±	0.13
	***P* value**	**66**		**0.001****			**0.66**			**0.24**			**0.97**	
Rhmani	CC	11	806.73^{a}	±	58.26	407.09	±	40.01	1.12	±	0.09	0.44	±	0.15
	CT	18	855.01^{a}	±	40.32	377.36	±	26.39	0.98	±	0.06	0.52	±	0.10
	TT	12	960.95^{b}	±	56.85	300.28	±	37.71	0.99	±	0.08	0.65	±	0.15
	***P* value**	**41**		**0.04***			**0.06**			**0.24**			**0.39**	
Barki	CC	12	579.99	±	18.30	347.57	±	51.25	1.00	±	0.00	1.00	±	0.00
	CT	7	617.47	±	18.24	364.68	±	45.79	1.00	±	0.00	1.00	±	0.00
	TT	0	-----	±		------	±	---	-----	±	----	-----	±	----
	***P* value**	**19**		**0.06**			**0.74**			**.**			**.**	
Within All Breeds	***P* value**	**126**		**0.04***			**0.59**			**0.10**			**0.93**	

Table (4): Effect of SNP of MTNR1A gene in Egyptian sheep breeds with first conception season on reproductive traits.

Genotype	FCS	Age at First Lambing(AFL)				Lambing Interval (LI)				Litter size (LS)				Fertility			
		N	LSM	±	SE	N	LSM	±	SE	N	LSM	±	SE	N	LSM	±	SE
CC	**LF**	39	832.09	±	45.11	20	419.45	±	49.51	27	0.96	±	0.05	39	0.68	±	0.10
	UF	54	709.72	±	42.31	21	523.37	±	50.76	33	1.03	±	0.04	54	0.67	±	0.10
	EF	42	698.87	±	44.31	21	371.16	±	48.18	25	0.95	±	0.05	42	0.66	±	0.10
CT	**LF**	24	808.65	±	49.29	11	370.96	±	57.32	16	0.96	±	0.05	24	0.74	±	0.11
	UF	64	692.97	±	41.61	20	505.05	±	50.33	35	1.02	±	0.04	64	0.68	±	0.10
	EF	65	742.70	±	39.63	37	430.48	±	50.33	40	0.96	±	0.04	65	0.66	±	0.10
TT	**LF**	9	716.30	±	82.49	3	331.49	±	111.02	3	1.42	±	0.11	9	0.62	±	0.19
	UF	30	915.82	±	51.46	11	396.68	±	60.51	17	0.94	±	0.05	30	0.71	±	0.12
	EF	11	924.00	±	73.21	5	411.57	±	81.57	7	0.93	±	0.07	11	0.69	±	0.17
	***P* value**	**0.01***				0.41				**0.002****				0.98			

Where LF: Late favorable, UF: unfavorable, EF: early favorable.

Genotyping and allelic frequency for AA-NAT gene

The allelic and genotypic frequencies of AA-NAT SNP 486A>G are shown in Table (2). The allelic frequency for Ossimi, Rhmani and Barki breeds was 0.258, 0.537 and 0.421 respectively for allele G; 0.742, 0.463 and 0.579 respectively for allele A. Also, genotype frequency for Ossimi, Rhmani and Barki breeds was 0.090, 0.244 and 0.0 respectively for genotype GG; 0.333, 0.585 and 0.842 respectively for genotype GA and 0.576, 0.171 and 0.158 respectively for genotype AA. In all tested samples the A allele showed high frequency (0.627) and heterozygous genotype GA has higher frequency (0.492). The observed and expected genotype frequencies were approximately the same. Results of the genetic diversity estimated by UHe showed highest degree in Rhmani (0.503) followed by Barki (0.501) and Ossimi (0.385). Result of overall populations was in accordance with the Hardy-Weinberg distribution. However, Barki breed was found to deviate from it (P=0.002).

Impact of MTNR1A genetic polymorphism on sheep reproductive traits

Results of association analysis of melatonin SNP 606C>T with reproductive traits revealed that days to first lambing was significantly lower for individuals carrying CT genotype in Ossimi breed and individuals carrying CC genotype in Rhmani breed. On the other hand, in Barki breed there were no significant differences between the two detected genotypes and all studied traits. Regarding to whole studied populations there were significant differences (P=0.04) with the detected genotypes for the age of first lambing while there were no significant differences with other investigated traits Table (3).The effect of SNP of MTNR1A gene in the sheep with first conception season on reproductive traits is presented in Table (4). There were significant differences in terms of age of first lambing (P < 0.01) and litter size (P =0.002), where the table demonstrates that ewes carrying CT genotype and conceive in UF season have the lowest mean measurements for age of first lambing followed by CC genotype conceiving in EF season. With reference to the litter size TT individuals seem

to be more intended for higher litter size in LF season followed by CC individuals in UF season. Regarding LI and fertility rate the result revealed no significant difference could be obtained.

Table (5): Effect of SNP of AA-NAT gene on reproductive traits in Egyptian sheep breeds.

Breed	Genotype	N	Age at First Lambing(AFL)			Lambing Interval (LI)			Litter size (LS)			Fertility		
			LSM	±	SE	LSM	±	SE	LSM	±	SE	LSM	±	SE
Ossimi	GG	6	933.68[a]	±	62.02	550.94[b]	±	72.80	1.20[a]	±	0.05	0.77	±	0.13
	GA	22	1072.31[b]	±	41.31	580.43[b]	±	48.44	1.05[b]	±	0.04	0.48	±	0.09
	AA	38	987.04[ab]	±	31.63	432.29[a]	±	32.43	1.01[b]	±	0.03	0.61	±	0.07
	***P* value**	**66**		**0.02***			**0.01***			**0.01***			**0.08**	
Rhmani	GG	10	783.98	±	56.80	359.34[a]	±	38.51	0.94	±	0.08	0.55	±	0.14
	GA	24	899.06	±	41.00	354.80[a]	±	24.24	1.03	±	0.06	0.57	±	0.10
	AA	7	882.34	±	69.71	522.39[b]	±	57.53	0.97	±	0.13	0.21	±	0.17
	***P* value**	**41**		**0.14**			**0.02***			**0.54**			**0.09**	
Barki	GG	0	-------	±	-------	-----	±	-----	------	±	-------	----	±	-----
	GA	16	601.66	±	15.85	353.97	±	40.64	1.00	±	0.00	1.00	±	0.00
	AA	3	559.03	±	32.04	407.23	±	78.75	1.00	±	0.00	1.00	±	0.00
	***P* value**	**19**		**0.16**			**0.47**			**.**			**.**	
Within All Breeds	***P* value**	**126**		**0.03***			**0.45**			**0.35**			**0.22**	

Table (6): Effect of SNP of AA-NAT gene in Egyptian sheep breeds with first conception season on reproductive traits.

Genotype	FCS	Age at First Lambing(AFL)				Lambing Interval (LI)				Litter size (LS)				Fertility			
		N	LSM	±	SE	N	LSM	±	SE	N	LSM	±	SE	N	LSM	±	SE
GG	**LF**	2	676.16	±	155.24	1	247.31	±	175.81	2	0.96	±	0.12	2	1.18	±	0.36
	UF	22	662.37	±	57.94	8	377.90	±	79.20	13	1.14	±	0.06	22	0.81	±	0.13
	EF	22	578.54	±	56.84	10	378.05	±	64.25	12	0.96	±	0.06	22	0.68	±	0.13
GA	**LF**	45	759.96	±	39.12	24	403.43	±	42.21	31	1.04	±	0.04	45	0.71	±	0.09
	UF	71	806.94	±	39.15	25	453.08	±	42.94	44	1.06	±	0.04	71	0.68	±	0.09
	EF	52	796.07	±	42.55	29	410.00	±	44.34	30	1.00	±	0.04	52	0.59	±	0.10
AA	**LF**	25	873.19	±	52.93	9	358.71	±	65.34	13	0.98	±	0.06	25	0.64	±	0.12
	UF	55	669.77	±	44.04	19	499.15	±	51.31	28	1.00	±	0.05	55	0.62	±	0.10
	EF	44	722.56	±	45.43	24	344.06	±	48.35	30	0.98	±	0.04	44	0.73	±	0.10
	***P* value**		**0.003****				0.37				0.39				0.33		

Impact of AA-NAT genetic polymorphism on sheep reproductive traits

Association analysis of AA-NAT SNP 486A>G between breed on reproductive traits are shown in Table (5). The result showed that Ossimi breed had significant differences between genotypes and reproductive traits in term of AFL (P= 0.02) and LS (P<0.01). Where, GG Ossimi individuals have the lowest AFL and the highest LS. On the other hand, AA Ossimi individuals showed significantly (P<0.01) lower lambing interval days. For Rhmani breed the least square means showed that GA individuals had the fewest days (354.80) for lambing interval (P=0.02). Regarding to Barki breed there were no significant differences between different genotype and traits. Regarding whole studied populations there was significant difference (P=0.03) for the age of first lambing only.

The results of association analysis of SNP of AA-NAT gene in the sheep with first conception season and reproductive traits are presented in Table (6). A significant difference (P=0.003) was detected with age of first lambing, where individuals carrying GG genotype and conceive in EF season or even conceive in UF season have fewer days to reach the age of first lambing. With reference to rest of reproductive traits there is no significant difference could be detected.

DISCUSSION

Seasonal reproduction in sheep is the primary factor that limits its economic production. The effect of seasonality on reproduction in small ruminants can be limited by implementing MAS selection programs using genetic markers. Genes are selected to either increase the ovulation rate or eliminate the limiting effect of seasonality (Trecherel *et al.,* 2010). The discovery of quantitative trait loci and their use in MAS selection could enhance selection responses significantly (Hristova *et al.,* 2012). MTNR1A is thought to be the main receptor involved in the regulation of seasonal reproductive activities in mammals (Dubocovich *et al.,* 2003). Moreover, MTNR1A gene polymorphism has been found to be significantly related to seasonal reproduction in sheep (Carcangiu *et al.,* 2009[a]), goats (Carcangiu *et al.,* 2009[b]), and buffalo (Carcangiu *et al.,* 2011). Also, AA-NAT which is a key rhythm-generating enzyme of the melatonin synthesis (Chattoraj *et al.,* 2009) can be incriminated in animal seasonal breeding (Mingxing, *et al.,* 2013). In the present study successful amplifications of 824bp and 1142bp of MTNR1A and AA-NAT genes respectively were achieved. The amplicons were digested with Rsa I and SmaI enzymes for detection of SNP 606 C>T and 486 A>G of MTNR1A and AA-NAT genes respectively. Results of PCR-RFLP revealed three genotypes (CC, CT and TT) for exon II of MTNR1A gene and (GG, GA and AA) for exon III of AA-NAT gene and two alleles were detected for each gene. The allelic frequency of MTNR1A gene obtained in this study revealed that allele C was predominant in both Ossimi and Barki breeds. This result is in accordance with the previous study of Mura *et al (*2014) on Sarda sheep, Moradi *et al.* (2014) on Zel and Naeini lamb and Giantsis *et al.* (2016) in local Greek sheep breed. Quite different result obtained in Rhmani breed where allele T was predominant. Similar results were recorded by Mateescu *et al.* (2009) in Dorest sheep and by Saxena *et al. (*2015) in sub-temperate sheep breed. Concerning to genotypic distribution of melatonin gene the CT had high frequency in Ossimi, Rhmani and CC genotypes was more frequent in Barki breed. The TT genotype had the lowest frequency in Ossimi and not

detected in Barki although was high in Rhmani breed. Mura *et al.* (2010) reported that the CC genotype had high frequencies in studied breeds while the TT genotype frequency was predominant in Dorset ewes) (Mateescu *et al.,* 2009). Absence of TT genotype in Barki breed in the present study might be due to the low sample size or for the lowest frequency of T alleles in the present study. The same observation was reported previously by Hristova *et al.* (2012) on Bulgarian LKNB sheep breed. Heterozygosity at RsaI marker site revealed high genetic variability in both Ossimi and Rhmani sheep populations. This result is in accordance with previous results of Moradi *et al. (*2014) on Zel and Naeini ewes. Results of allelic distribution of AA-NAT gene revealed that A allele was more frequent in Ossimi and Barki sheep while G allele was more frequent in Rhmani sheep. The same allelic distribution was obtained by Ding-Ping *et al.* (2012). Regarding to genotypic distribution of AA-NAT gene, GA and AA genotypes were detected in the three sheep breeds under investigation. However, GG genotype was not observed in Barki sheep. Due of rarity of literature on AA-NAT gene polymorphism in sheep, a kind of difficulty has been arisen to compare our results on Egyptian sheep with others. According to the results reported in Table (3) there are significant correlation between different genotypes and AFL in Rhmani and Ossimi breeds where CC Rhmani and CT Ossimi had the lowest mean and allele C seems to be the favorable allele. Similarly, Mura *et al.,* (2014) reported that ewes carrying CC and CT genotype had significantly higher fertility rate. In the same sequence the allele C exhibited a better reproductive efficiency as reported by several research articles (Notter *et al.,* 2003; Chu *et al.,* 2006 and Carcangiu *et al.,* 2009[a]). On the other hand, Barki sheep investigated in this work showed no significant difference between genotypes and age of first lambing. The same observation was reported by Mateescu *et al.* (2009) on Dorest ewe. Investigation of AA-NAT SmaI marker pointed out that GG Ossimi ewes were more favorable for lower AFL and higher litter size. However, GA Rhmani sheep should be taken into account to be selected for lower LI followed by AA Ossimi sheep could be selected for the same trait. For Barki ewe no significant effect of genotypes on reproductive traits was obtained and more investigation must be carried out. Results of Table (4) demonstrates that ewes carrying CT genotype and conceive in UF season have the lowest mean measurements for age of first lambing. This result is in accordance with Carcangiu *et al (*2009) [b] who showed a strong link between heterozygous genotype and reproductive activity in different goat breed. It could be explained as ewes that conceive in the UF season are more likely to conceive sooner after lambing because their next breeding season will be in EF season (Mateescu *et al.,* 2009). Or can be hypothesized that the ewes carrying one or more C alleles exhibit a shallow state of anoestrus that leads to their higher response to the ram effect (Carcangiu *et al.,* 2012). This supposition is in accordance with previous studies in other sheep breeds (Carcangiu *et al.,* 2009[a] and Chu *et al.,* 2006). So, ewes that carrying one or more C allele are seem to more desirable or selectable for the lower age of first lambing. With reference to litter size it is obvious that sheep carrying TT genotype had greater litter size in LF season. Likewise, Chu *et al.* (2003) reported that Small Tail Han ewes with TT genotype had greater litters size especially in second parity. As for association with SNP in AA-NAT gene with reproductive traits and season (Table 6) the result found out that GG ewes which conceive in EF season or even conceive in UF season have fewer days to reach the age of first lambing. This

observation is in agreement with the previous results of Ding-Ping *et al.,* (2012), who suggested that the genotype GG might be associated with superior unseasonal reproduction. It is definitely from the obtained data that ewes carrying CT or GG genotypes could be allocated for reproduction during long photoperiod instead of the TT or AA genotype in natural mating season.

CONCLUSION

The present study confirmed presence of genetic polymorphisms in MTNR1A and AA-NAT loci in Egyptian Ossimi, Rhmani, and Barki sheep breed. The results pointed out a significant relationship between MTNR1A and AA-NAT loci and reproductive traits AFL, LI, LS. This data proves importance of both loci and their polymorphisms could be measured as possible genetic markers suitable for improving efficiency of reproduction during unfavorable (UF) non-breeding long photoperiod season in sheep and the obtained desirable genotypes could be taken into account in new genetic selective schemes as safe alternative way to hormonal treatment for reduction of seasonality. According to personal knowledge this is the first study concerned with polymorphisms in MTRN1A-RsaI and AA-NAT-SmaI loci and their association with reproductive traits in Egyptian sheep breeds. Further studies are required to validate the association in larger scale population especially in Barki breed and its influence on other economically important traits should be surveyed.

Conflict of interest

The authors declare that they have no Conflict of interest.

ACKNOWLEDGEMENT

The authors would like to thank the members of Biotechnology unit in Animal reproduction research institute for their great help and encouragement, and we acknowledge the contribution of other staff who helped in obtaining the samples and reproductive data.

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الملخص العربي

التأثير المحتمل لتعدد الطرز الجينية لجين مستقبلات الميلاتونين 1A و جين الأريل آلكيل أمين ن أسيتيل ترانسفيريز علي موسمية التكاثر في سلالات الأغنام المصرية

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أجريت هذه الدراسة للكشف عن التعدد في الطرز الجينية لجين مستقبلات الميلاتونين 1A (MTNR1A) وجين الأريل ألكيل أمين ن أسيتيل ترانسفيريز (AA-NAT) وارتباطها بالصفات التناسلية. تم تجميع عدد ١٢٦ عينة دم من من ثلاثة سلالات من الأغنام المصرية. تم إستخلاص الحامض النووي من العينات وأجري عليه إختبار إنزيم البلمرة المتسلسل والتقطيع بإستخدام إنزيمات القطع الجيني SmaIوRsaI. تم الكشف عن أثنين من الأليلات (C,T) وثلاثة تراكيب وراثية (CC, CT, TT) بالنسبة لجين مستقبلات الميلاتونين 1A وأيضا أليليلن (A, G) و ثلاثة تراكيب وراثية (GG, GA, AA) بالنسبة لجين الأريل ألكيل أمين ن استيل ترانسفيريزز. أظهرت الأليلات (C, A) والتراكيب الوراثية (CT, GA) أعلي نسبة تواجد بالنسبة لجين مستقبلات الميلاتونين 1A وجين الأريل ألكيل أمين ن استيل ترانسفيريز علي التوالي. كشفت تحاليل الأرتباط لجين مستقبلات الميلاتونين 1A عن وجود إرتباط معنوي في سلالات الأوسيمي والرحماني مع العمر عند أول ولادة فيما يبدو أن الأليل C هو الأليل المميز. كما أظهرت نتائج تحليل الإرتباط لجين الأريل ألكيل أمين ن استيل ترانسفيريز فروقًا معنوية في سلالة الأوسيمي بالنسبة للعمر عند أول ولادة و حجم الولادات بينما كانت هناك فروقًا معنوية في سلالة الرحماني بالنسبة للفترة بين ولادتين و فيما يبدو أن الأليل G هو الأليل المرغوب. وفيما يتعلق بموسم الحمل الأول ، كشفت الدراسة أن النعاج التي تحمل التركيب الوراثى CT أظهرت انخفاض كبير في العمر عند أول ولادة في الموسم غير المفضل. كما أظهرت النعاج GG أقل سن للعمر عند أول ولادة في أوائل موسم التناسل المفضل يليها موسم التناسل الغير مفضل للتناسل.وفقا للمعرفة الشخصية ، فإن هذه الدراسة تعد هي الدراسة الأولى المعنية بهذا الارتباط في سلالة الأغنام المصرية. الخلاصة: يمكن قياس تعدد الأشكال التي تم تحقيقها في هذه الدراسة كواسمات وراثية مناسبة لتحسين الكفاءة التناسلية خلال الموسم غير المواتي ويمكن أخذ الأنماط الجينية المرغوب فيها في الاعتبار في مخططات انتقائية وراثية جديدة.