

Polymorphisms of the melatonin receptor 1A (*MTNR1A*) gene affect the sexual performance of Rasa Aragonesa rams without changing their social dominance



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Abstract This study aimed to investigate whether a specific genotype of the *MTNR1A* gene influences social dominance and reproductive performance in rams. Thirty-one rams were genotyped for RsaI and MnlI polymorphism of the gene: CC (n=19), CT (n=5), and TT (n=7), and GG (n=20), GA (n=6), or AA (n=5), respectively. Maintenance (standing still, lying down, drinking, feeding, walking, or stereotyping) and social behavior (agonistic and antagonistic interactions among rams) were recorded by direct observations involving instantaneous scan sampling and continuous behavior sampling. For each animal, his Index of Success (IS) and Displacement (ID) were calculated. One week after the social-behavioral observations, rams were tested in individual serving-capacity tests involving three ewes that were synchronized into estrus in a 15-m² pen for 20 min. The genotypes did not differ in the number of aggressions they performed (CC: 88.95±12.85; CT: 106.20±21.00; TT: 70.43±8.50; GG: 76.05±8.72; GA: 119.00±16.16; AA: 95.80±36.39) or received (CC: 75.79±5.82; CT: 92.40±8.68; TT: 86.14±15.05; GG: 79.25±6.58; GA: 86.83±11.51; AA: 79.80±12.74), and their active and passive behaviors were similar. The genotypes did not differ significantly in IS or ID, and the proportion of rams in each success category was similar among groups. For the RsaI and MnlI polymorphism, TT and GG rams were responsible for 54% and 56% of recorded sexual events ($P < 0.05$), respectively. Results of this study confirm that the best sexual performance of rams was among those that carried certain genotypes of the *MTNR1A* gene. Still, it was not correlated with differences in social dominance.

Keywords: melatonin, receptor, sexual behavior, sheep, social dominance

1. Introduction

Domestic sheep live in large groups, display intensely gregarious behavior, and develop stable social relationships with other group members (Ozella et al 2020). Sociality is an adaptive strategy and is part of the complex and dynamic environment of the individual in which many strategies have evolved to improve survival and maintain the viability of the flock (Miranda de la Lama and Mattiello 2010). Group living, however, has biological costs caused by competition and conflict (Silk 2007). Social competition between animals is regulated primarily through dominance (Val-Laillet et al 2008). Social dominance can be defined as a priority of access to an approach situation or away from an avoidance situation that one animal has over another (Francis 1988). Competitive ability, experience, personality, morphology, and kinship can influence dominance (Miranda-de la Lama et al 2019; Hobson 2020). From an energetic perspective, dominance rank

requires a large initial investment (displays, fights, persecution, and exclusion of some individuals) that pays dividends by reducing investment in energetically expensive aggression related to resources such as shelter, food, space, and mates. Subordinate individuals use fewer desirable resources to avoid fights with dominant opponents (Lord et al 2021). Probably, genetic factors and ontogeny contribute to any predisposition toward susceptibility to social stress and its effects on reproduction (Larrieu and Sandi 2018).

Photoperiod is a seasonal cue that predicts changes in resource availability and environmental conditions and affects reproduction and social interactions in many mammals (Laredo et al 2014). Photoperiod is the main regulator of seasonal reproduction in sheep (Arendt 1998), whose reproductive activity is marked by changes in the duration of day and night, which results in an anestrus season and a reproductive season (Hafez 1952). The animals conceive in autumn-winter, and births occur in winter-spring.

Annual variation in photoperiod, which regulates melatonin secretion, is the main environmental factor influencing reproductive seasonality. The higher melatonin concentrations at night, which typically occur in autumn, positively affect the reproductive activity of small ruminants (Bittman and Karsch 1984). Melatonin acts through specific receptors in various body organs, including the nuclei of the central nervous system that control reproductive activity (Sliwowska et al 2004). Two high-affinity melatonin receptors, MT1 and MT2, have been identified in mammals; however, MT1 is the one that is involved in the regulation of reproduction (Weaver et al 1996; Dubocovich et al 1998). There are many receptors in the pars tuberalis, but it has a limited role in regulating reproduction, and it is important in the photoperiodic control of prolactin secretion (Lincoln and Clarke 1994). Melatonin receptors occur in other tissues and cells, including male and female reproductive tracts (Dubocovich and Markowska 2005) and gametes (Casao et al 2012). The MT1 receptor, encoded by the *MTNR1A* gene, is part of the family of G-protein coupled receptors and has been found in several species (Messer et al 1997; Reppert et al 1994). In particular, two polymorphisms (Rsal and MnlI) in exon II of the *MTNR1A* gene affect seasonal reproduction in sheep (Pelletier et al 2000; Carcangiu et al 2009; Luridiana et al 2015a).

The *MTNR1A* gene polymorphisms and their association with ovulation seasonality in sheep (Pelletier et al 2000) are one of the most well-studied genetic factors. They have the potential to have positive effects on the profitability of farms. The gene has two exons separated by a long (8000 bp) intron (Carcangiu et al 2009) and causes females to exhibit estrous activity in the seasonal anestrus. In the Sarda breed, the presence of the *MTNR1A* genotype and the reproductive response after estrous synchronization and artificial insemination in spring are correlated, and an allelic variant of the receptor is associated with the reproductive response to the male effect (Mura et al 2019). Specifically, in that study, the g.15099485A> G MnlI variant had a positive impact on the timing of the cessation of seasonal anestrus when the rams were introduced to the ewes in March or April. Furthermore, the association between the SNPs g.15099485A> G and g.15099391G> A might help to identify the mechanism of melatonin's action and its receptors' role in reproductive seasonality. In addition, in the Rasa Aragonesa breed, the presence of the T allele of Rsal polymorphisms of the *MNTR1A* gene is associated with a shortened anestrus period and more complete ovarian cycles per year (Calvo et al 2018). In ewes of that breed (Martínez-Royo et al 2012) and Slovenian sheep (Starič et al 2020), the TT genotype and the proportion of ovarian cyclicity that occurred between January and August was strongly correlated. Similarly, in Sarda ewes, animals that have the GG genotype seem to have a higher number of ewes ovulating and ovarian cyclicity throughout the year and a better reproductive response to treatment with melatonin or progestogens than do other genotypes (Luridiana et al 2015a).

Information on the effects of those polymorphisms on the ram is limited. Polymorphisms of the *MTNR1A* gene sequence can influence the reproductive performance of young and adult rams in spring (Abecia et al 2020). Specifically, Rasa Aragonesa ram lambs born in autumn that carry the TT or GG genotypes are more likely to reproduce in their first spring, and adult TT or GG rams exhibit more intense reproductive behavior as measured by a sexual behavior test in spring. The main conclusion from that preliminary study was that genotyping those animals can be a useful procedure toward a more correct and rational use of rams in sheep farming. Therefore, this study aimed to investigate whether carrying a specific genotype of the *MTNR1A* gene influences social dominance in Rasa Aragonesa rams and whether it is correlated with an improvement in reproductive performance.

2. Materials and Methods

The experiment was conducted at the experimental farm of the University of Zaragoza, Spain (41°38'05.7"N 0°51'36.8"W; 199 msnm), following protocols approved by the Ethics Committee for Animal Experiments of the University of Zaragoza. The care and use of animals were following the Spanish Policy for Animal Protection (RD 53/2013), which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

2.1. Animals

Thirty-one adult Rasa Aragonesa rams [3.5 years old, mean (\pm S.D.) live weight (LW) = 98.2 \pm 8 kg] were housed for 12 months in a communal pen (15x6 m²) that had a similarly sized outdoor area (5.8 rams/m²) to ensure a stable flock social structure. Rams and ewes were fed to meet their LW maintenance requirements and had unlimited access to water and mineral salts. Rams were genotyped for their Rsal and MnlI polymorphism of the *MTNR1A* gene as follows: genotype CC (n=19), CT (n=5), and TT (n=7) for the Rsal allele, and GG (n=20), GA (n=6), or AA (n=5) for the MnlI allele.

2.2. Social behavior measurements

In mid-June, a trained observer sat on a 3-m-high platform and recorded information on the maintenance and social behaviors of the rams (Martin et al 1993) through direct observations that involved instantaneous scan sampling and continuous behavior sampling. To identify each ram, large (30-cm-high) numbers were painted on the sides and rump of the males with washable sheep spray paint. The same person always observed the rams simultaneously for 6 h (0700-0900 h, 1100-1300 h, and 1600-1800 h) on 16 consecutive days.

Continuous behavior sampling was used between the maintenance behavior sampling episodes to record agonistic interactions among rams. Agonistic interactions with contact included butting (a ram used the front of his head to make contact with another ram), pushing (a ram used other parts

of his body to make contact with another ram), mounting (a ram straddled and clasped another ram, contacting his brisket with the other ram's rump), and kicking (a ram kicked the other ram with his front legs). Agonistic interactions without contact included threatening (a ram with his head down turned toward or approached another ram and lunged without making contact), chasing (a ram purposely moved toward another ram causing the latter to walk or run away), and avoidance (a ram purposely moved away from another individual in response to an earlier agonistic interaction).

Two indices of social behavior were calculated to estimate the social status of each ram based on his experiences in aggressive interactions; viz., the Index of Displacement (ID) and the Index of Success (IS). ID was calculated as follows: ID = number of times a ram displaced another ram/(number of times he displaced another + number of times he was displaced).

For each animal, the number of times he engaged in each of those social interactions was recorded, and the IS was calculated based on the data collected on interactive behaviors that reflected the social status of each ram (Mendl et al., 1992) based on his experiences in agonistic interactions with other members of the flock. The IS ranges from 0 to 1, and is calculated as follows: IS = number of individual rams he displaced/(number of individual rams he displaced + number of individual rams that displaced him). Rams were assigned to one of three ranking categories based on their ID and IS as follows: low (0.0-0.33), medium (0.34-0.66), and high (0.67-1.0) (Miranda-de la Lama et al 2011).

2.3. Serving-capacity test

One week after the social-behavioral observations, five rams of each genotype were tested in individual serving-capacity tests (Kilgour and Whale 1980; Damián et al 2015) that involved 30 ewes that had been synchronized into estrus by intravaginal pessaries for 12 d and 300 IU eCG i.m. (SyncroPart, CEVA Salud Animal, Spain). Individual rams were exposed to three estrous ewes in a 15-m² pen for 20 min, and the following was recorded: number of flehmen (elevating the head and upper lip in response to taste and odor of ewe urine or ambient odor), anogenital sniffing (sniff in the genital region of ewe), approaches (intensely rubbing, licking, or superficially nibbling the flank of the ewe), attempted mounting (stands behind the ewe and moves intending to copulate, with front legs in the air, but not placed safely on the ewe), and mounting (intrusion of the penis into the vagina of ewe with one or more thrusts and, thereby, ejaculation can occur, which is indicated by the backward elevation of the ram's head) (Calderón-Leyva et al 2018).

2.4. Blood sampling, DNA analysis and sequencing

The procedures to identify the allelic variants of individual rams based on whole blood are described by Carcangiu et al. (2009). Briefly, DNA was extracted by a genomic DNA extraction kit (NucleoSpin® Blood, Macherey-Nagel, Germany). Polymerase chain reaction (PCR) was

performed on 150 ng of genomic DNA from each ram and specific primers (Sigma Genosys Ltd., Pampisford, Cambs, UK) (Messer et al 1997). The PCR and digestion reactions were performed as previously described (Carcangiu et al 2009; Mura et al 2019). The PCR products for each genotype were sequenced by an Applied Biosystems 3730 DNA Analyzer (PerkinElmer Applied Biosystems, Foster City, CA, USA), and the sequences were aligned and compared with the ovine sequence GenBank U14109 and GCA_002742125.1. The homology searches were performed through BLAST (National Centre for Biotechnology Information: <https://blast.ncbi.nlm.nih.gov/Blast.cgi>). To align the sequences, the CLUSTALW tool was used (<http://www.genome.jp/tools-bin/clustalw>).

2.5. Statistical analysis

An ANOVA was used to detect statistically significant differences among genotypes in maintenance and social behaviors and the indices. The proportion of rams of each allele type in each success category, and differences among genotypes in the proportions of sexual events observed in the sexual-capacity trials, were assessed statistically by a X² test. A *P*-value ≤ 0.05 was considered statistically significant.

3. Results

3.1. Social dominance

The three genotypes of each polymorphism did not differ significantly in social dominance, so that CC, CT, and TT rams for the RsaI polymorphism and GG, GA, and AA rams for the MnlI polymorphism performed and received a similar number of aggressions with or without contact, and their active and passive behaviors were similar (Table 1). The genotypes did not differ significantly in either ID or IS (Figure 1), and the proportion of rams in each success category was similar among genotypes (Table 2).

3.2. Serving-capacity test

For the RsaI polymorphism, TT rams were responsible for 67% of the flehmen, 68% of the approaches, 52% of the mountings, and 54% of the total events (*P* < 0.05). Among the MnlI genotypes, GG rams performed a significantly (*P* < 0.05) higher proportion of the flehmen (63%), anogenital sniffing (55%), mounting attempts (55%), mountings (63%), and total activities (56%) (Figure 2).

4. Discussion

This study confirmed the results of an earlier study (Abecia et al 2020); specifically, an increase in the sexual performance in the spring of rams that carried specific genotypes of the *MTNR1A* gene, although it was not associated with the social hierarchy in the ram flock. Our study's genotypic and allelic frequencies were similar to those reported for the same breed (Calvo et al 2018). The two *MTNR1A* gene loci appeared to influence the reproductive behavior of Rasa Aragonesa rams. Specifically, the TT

genotype at position g.17355458 C>T, and the GG at position g.17355452G>A positively affected the sexual performance of rams. In female sheep, the TT genotype is associated with a high proportion of cyclic ewes in Rasa Aragonesa (Martínez-Royo et al 2012) and Slovenian ewes (Starič et al 2020), and the GG genotype, which seems to have the best reproductive recovery, ovulatory cyclicity throughout the year, and reproductive response to treatment with melatonin (Luridiana et al 2015a,b); however, as we have demonstrated in our study, rams that carried these genotypes did not have a social dominance that differed significantly from that of the other genotypes.

In intensive farming sheep systems, hierarchies often are bidirectional and nonlinear such that the social rank of a

sheep over another is not absolute, which is true in goats and cattle (Lynch et al 1992); however, intensive rearing conditions such as those in our study tend to exacerbate competition for valuable resources such as space, access to food, and resting places (Nowak et al 2008). ID and IS are commonly used in studies of social dominance and are complementary to each other because ID is useful for quantifying the frequency of flights between individuals because it weights the number of times that an individual displaces another, and IS weights the efficiency by identifying how many individuals in the group the individual can displace (Mendl et al 1992). However, our results show that the polymorphisms of the melatonin receptor 1A and the two dominance indices were not correlated.

Table 1 Mean (±S.E.) number of maintenance and social behaviors of Rasa Aragonesa rams carrying the Rsa1 (CC, CT, or TT) or Mnl1 (GG, GA, or AA) polymorphism of the *MTNR1A* gene, through direct observations, with a combination of instantaneous scan sampling and continuous behavior sampling methods.

	Rsa1 polymorphism			Mnl1 polymorphism		
	CC	CT	TT	GG	GA	AA
No.	19	5	7	20	6	5
Aggressions with contact						
Butting	19.32±4.27	25.00±10.02	13.86±2.82	16.45±2.97	27.33±10.51	19.20±9.26
Pushing	60.11±8.53	69.20±13.14	51.43±8.89	52.05±6.16	80.83±9.55	64.40±23.99
Mounting	2.37±0.96	3.40±1.63	1.57±1.11	2.00±0.89	4.17±1.70	1.60±1.03
Kicking	7.16±1.52	8.60±1.91	3.57±1.09	5.55±1.01	6.67±2.04	10.60±4.45
Total	88.95±12.85	106.20±21.00	70.43±8.50	76.05±8.72	119.00±16.16	95.80±36.39
Aggressions with no contact						
Threatening	5.11±1.09	5.20±1.46	3.86±1.39	4.15±0.71	6.17±2.07	6.00±3.08
Chasing	0.37±0.17	1.00±0.32	0.86±0.34	0.50±0.18	0.67±0.33	0.80±0.37
Total	5.47±1.14	6.20±1.59	4.71±1.27	4.65±0.72	6.83±2.09	6.80±3.25
Received aggressions with contact	75.79±5.82	92.40±8.68	86.14±15.05	79.25±6.58	86.83±11.51	79.80±12.74
Received aggressions with no contact	7.21±1.40	5.60±1.86	5.43±1.38	5.70±0.87	7.83±1.87	8.40±4.53
Active avoidance behavior	17.95±1.60	20.60±3.91	18.86±2.26	18.35±1.62	19.33±1.61	18.60±4.19
Passive avoidance behaviour	20.53±2.64	29.00±3.92	19.86±3.19	20.75±2.06	26.50±6.89	20.00±3.45
Total avoidance behavior	38.47±3.43	49.60±6.56	38.71±3.69	39.10±3.00	45.83±6.44	38.60±7.37
Ignore	12.79±1.44	12.00±1.95	20.71±11.26	14.05±4.01	15.83±3.07	14.40±1.72
Aggressive response with contact	36.32±2.95	39.80±5.40	33.71±3.27	33.70±2.11	40.33±4.80	41.80±8.22
Aggressive response with no contact	2.37±0.40	2.20±0.58	2.86±1.28	2.75±0.51	2.17±0.70	1.60±0.81
Initiates affiliative behaviors	35.21±8.02	46.00±6.73	39.57±10.27	29.75±5.71	61.67±17.75	42.20±7.68
Receive affiliative behaviors	28.26±3.86	24.80±8.71	73.57±51.64	41.45±18.21	27.17±6.36	36.80±9.83

Table 2 Proportion of low, medium, and high social rank, as measured by the Index of Success, of Rasa Aragonesa rams carrying the Rsa1 (CC, CT, or TT) or Mnl1 (GG, GA, or AA) polymorphism of the *MTNR1A* gene. Index of Success = number of individual rams he displaced/(number of individual rams he displaced + number of individual rams that displaced him).

	Social rank (by Index of Success)		
	Low	Medium	High
CC	32%	47%	21%
CT	40%	40%	20%
TT	0%	57%	43%
GG	30%	40%	30%
GA	33%	67%	0%
AA	0%	60%	40%



Social dominance is established and maintained through aggressive behaviors usually observed in dominant animals, and defensive and submissive behavior is observed in subordinate individuals (Wang et al 2012). Hormonal modulators, including testosterone, adrenal steroids, and melatonin, are responsible for much of the variance in aggressive animal behaviors (Liu et al 2017). Melatonin receptors appear to be important in learning and memory in mammals, and the variance in aggressive animal behaviors is caused by the effects of melatonin (Liu et al 2017). Both

endogenous and exogenous melatonin increase aggression in seasonal breeders (Jasnow et al 2002); however, the role of melatonin in increasing aggression is highly dependent on the social context of the animals (Laredo et al 2014). In our study, melatonin receptors did not affect social dominance in a socially stable group. Therefore, melatonin's role in aggressive behavior (contact or non-contact) may be activated through melatonin receptor-independent mechanisms.

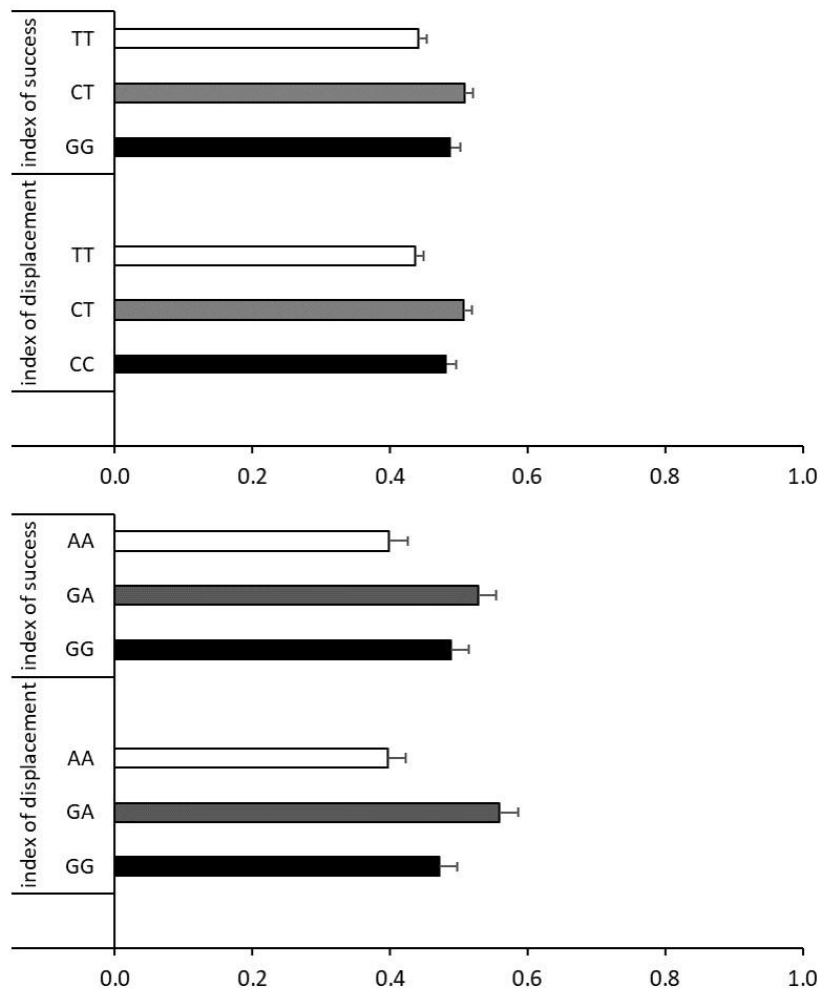


Figure 1 Mean (±S.E.) Index of Displacement and Index of Success of Rasa Aragonesa rams carrying the RsaI (CC, CT, or TT) or MnlI (GG, GA, or AA) polymorphism of the *MTNR1A* gene. Index of Displacement = number of times a ram displaced another ram/(number of times he displaced another + number of times he was displaced). Index of Success = number of individual rams he displaced/(number of individual rams he displaced + number of individual rams that displaced him).

In a study involving a flock of rams in which the hierarchy was well described (Ungerfeld and González-Pensado 2009), the number of ewes mated by high-ranking rams in an individual mating behavior test was lower than it was in low-ranking rams if tested alone. However, in competitive tests, low-ranking rams were more affected by the presence of dominant rams than dominant rams in the presence of submissive rams. This suggested no relationship between social rank and testis characteristics because submissive rams were more effective than dominant rams in mating ewes in the absence of competition, the condition of

the tests in our study. Possibly, that might be caused by the relaxation of natural selection (Price 1985). Under conditions of high competence in which sexual performance is under intense selection pressure, variation in measures of sexual performance would be expected to be relatively small, with most males exhibiting near-maximal sexual motivation and performance (Katz 2008). In contrast, the absence of competition that allows males with sub-standard sexual performance to reproduce might contribute to higher variance in this trait, which increases the number of males that exhibit poor performance.



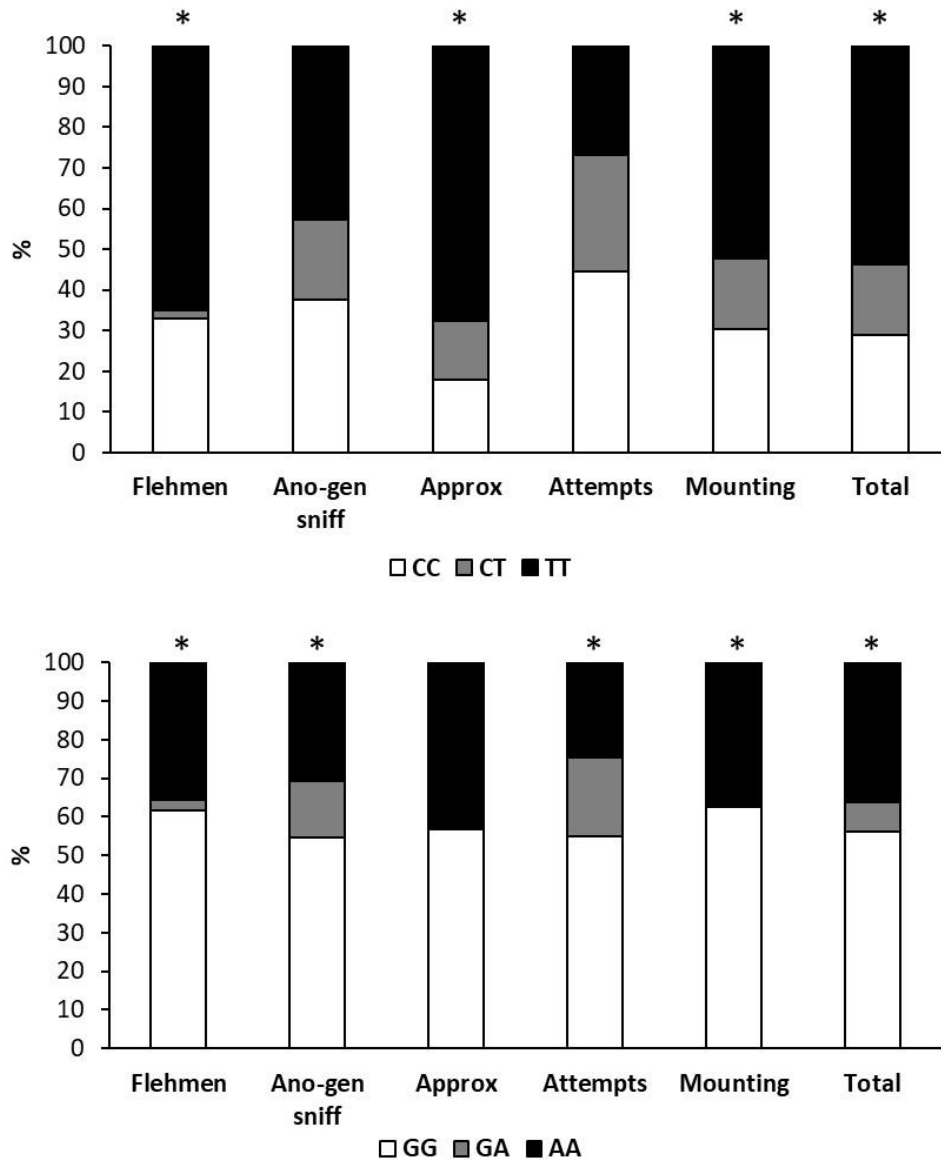


Figure 2 The proportion of flehmen, anogenital sniffing, approaches to the females, attempts of mounting, mountings, and total number of recorded events by Rasa Aragonesa rams carrying the Rsa1 (CC, CT, or TT) or Mnl1 (GG, GA, or AA) polymorphism of the *MTNR1A* gene, in a serving capacity test, individually exposed to three estrous ewes in a 15-m² pen for 20 min (*indicates $P < 0.05$).

5. Conclusions

Results of this study confirm the previously observed best sexual performance of rams carrying specific genotypes of the *MTNR1A* gene, although it was not associated with differences in social dominance.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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