Differences in the Ovine *HSP90AA1* Gene Expression Rates Caused by Two Linked Polymorphisms at Its Promoter Affect Rams Sperm DNA Fragmentation under Environmental Heat Stress Conditions

Judit Salces-Ortiz[1‡], **Manuel Ramón**[2*‡], **Carmen González**[1], **M. Dolores Pérez-Guzmán**[2], **J. Julián Garde**[3], **Olga García-Álvarez**[3], **Alejandro Maroto-Morales**[3], **Jorge H. Calvo**[4], **M. Magdalena Serrano**[1]

1 INIA, Carretera de La Coruña Km. 7.5, 28040, Madrid, Spain, **2** CERSYRA, Av. Del Vino 10, 13300, Valdepeñas, Spain, **3** SaBio IREC (CSIC–UCLM–JCCM) Campus Universitario S.N., 02071, Albacete, Spain, **4** Unidad de Tecnología en Producción Animal, CITA, 59059, Zaragoza, Spain

* m.ramon.fernandez@gmail.com
‡ These authors contributed equally to this work.

Abstract

Heat shock (HS) is one of the best-studied exogenous cellular stresses. Almost all tissues, cell types, metabolic pathways and biochemical reactions are affected in greater or lesser extent by HS. However, there are some especially thermo sensible cellular types such as the mammalian male germ cells. The present study examined the role of three INDELs in conjunction with the -660G/C polymorphism located at the *HSP90AA1* promoter region over the gene expression rate under HS. Specially, the -668insC INDEL, which is very close to the -660G/C transversion, is a good candidate to be implied in the transcriptional regulation of the gene by itself or in a cooperative way with this SNP. Animals carrying the genotype II-668 showed higher transcription rates than those with ID$_{-668}$ (FC = 3.07) and DD$_{-668}$ (FC = 3.40) genotypes for samples collected under HS. A linkage between gene expression and sperm DNA fragmentation was also found. When HS conditions were present along or in some stages of the spermatogenesis, alternative genotypes of the -668insC and -660G/C mutations are involved in the effect of HS over sperm DNA fragmentation. Thus, unfavorable genotypes in terms of gene expression induction (ID$_{-668}$GC$_{-660}$ and DD$_{-668}$GG$_{-660}$) do not produce enough mRNA (stored as messenger ribonucleoprotein particles) and Hsp90α protein to cope with future thermal stress which might occur in posterior stages when transcriptional activity is reduced and cell types and molecular processes are more sensible to heat (spermatocytes in pachytene and spermatids protamination). This would result in the impairment of DNA packaging and the consequent commitment of the events occurring shortly after fertilization and during embryonic development. In the short-term, the assessment of the relationship between sperm DNA fragmentation sensitivity and ram's fertility will be of interest to a better

understanding of the mechanisms of response to HS and its consequences on animal production and reproduction performance.

Introduction

Heat shock (HS, also known as heat stress or hyperthermia) is one of the best-studied exogenous cellular stresses. The cellular response to HS utilizes ancient molecular networks that are based primarily on the action of stress-induced heat shock proteins (HSP) and heat shock factors. All eukaryotes produce at least one heat inducible protein with molecular sizes that ranges from 80 to 90 kDa [1]. Members of the *HSP90* gene family have undergone major duplication events, which led to two known cytoplasmic isoforms, namely Hsp90α (also named *Hsp90AA1*) or inducible form and Hsp90β (also named *Hsp90AB1*) which is the constitutive one. Whereas Hsp90β is more or less constitutively and ubiquitously expressed, the expression of Hsp90α is heat-inducible and more tissue specific [2]. Hsp90α predominates in the brain and testis and is necessary for the spermatogenesis and meiotic progression in testis [3]. The transcriptional enhancement of Hsp90α is mainly due to the HSF1 (heat shock factor 1), but many other heat stress related transcription factors also regulate gene expression in response to environmental stress [1, 4].

Almost all tissues, cell types, metabolic pathways and biochemical reactions are affected in greater or lesser extent by heat stress. However, there are some cellular types specially thermo sensible such as the mammalian male germ cells. The sensitivity of these cells to environmental heat has been extensively studied [5–7]. A reduction in sperm DNA integrity has been described in human [8], mice [9, 10] and rams [11] as well as alterations in DNA, RNA and protein synthesis, and abnormal chromatin packing in mice [9, 10, 12] under heat stress conditions. Proper compaction and structure of DNA has been reported to have important functional roles, being essential for DNA replication and embryonic development [13, 14]. In a recent work of our group [15] it has been observed that sperm DNA fragmentation in rams increases over a threshold of 30°C of maximum temperature (temperature-humidity index THI > 22) at some stages of the spermatogenesis process in animals carrying the GG_{-660} genotype of the -660G/C SNP (rs397514116). As GG_{-660} genotype has been associated to lower levels of *HSP90AA1* expression under heat stress conditions [16] it has been suggested that suboptimal amounts of *HSP90AA1* mRNA make spermatozoa DNA of these animals more susceptible to be fragmented.

In sheep, the gene encoding the *HSP90AA1* isoform has been extensively studied [17–19]. At the promoter of this gene 14 polymorphisms (11 SNPs and 3 INDELs) have been described [17, 19]. One of these mutations, -660G/C SNP (rs397514116), have been related with the *scrapie* incubation period [18]; the adaptation of this species to differential thermal conditions [20]; and as noted above, ram's sperm DNA fragmentation levels [15]. All these facts, which have been observed under thermoneutral, heat stress and missfolding stress conditions, seem to be consequence of the differences in the gene expression profile caused by this SNP located at the gene promoter [16, 20]. Therefore, it is reasonable to hypothesize that these polymorphisms are affecting some mechanisms of the gene transcription regulation (transcription factor binding affinity, structural features, etc) leading to differences in the RNA synthesis efficiency. However, as -660G/C is in linkage disequilibrium with other close mutations [16] it is not possible to determine which is/are the causal mutation/s responsible/s of the expression differences observed. In addition, until today, the possible relation with the expression rate of the gene of the three INDELs at positions -667insC, -668insC and -516insG (rs397514115.1,

rs397514115.2 and rs397514268, respectively) located at the *HSP90AA1* gene promoter have been never studied.

The present study was designed to better understanding the role of the *HSP90AA1* gene in the response of animals to heat stress. Specifically, we aimed to know more about the expression patterns of the gene and the consequences in sperm DNA fragmentation levels derived from an exposure to a heat stress environment and depending on the genotype of males for the -660G/C SNP and the three INDELs located at the *HSP90AA1* gene promoter. For that, this work included two specific objectives: 1) study the effect of some INDELs located at the *HSP90AA1* gene promoter over its expression rate under heat stress and control conditions by q-PCR; 2) Test the effect of the INDELs along with the -660G/C SNP over ram's sperm DNA fragmentation under different environmental conditions.

Material and Methods

Ethics Statement

The current study has been approved by the INIA Scientific Ethic Committee (IACUC). Animal manipulations were performed according to the Spanish Policy for Animal Protection RD 53/2013, which meets the European Union Directive 63/2010/EU about the protection of animals used in experimentation.

Weather data

As a way to examine the role of -660G/C and -668insC polymorphisms on the observed effect of heat on sperm chromatin integrity [15, 16], climate and sperm DNA status data were gathered from March to October.

Meteorological data was provided by the Irrigation Advisory Service for Farmers (SIAR) in Castilla-La Mancha. Table 1 shows climate parameters at day of blood and sperm samples collection, and also the average from 2 days before to the day of samples collection. The meteorological data set consisted of hourly measures of temperature (°C) and relative humidity (%) on 245 days from March to October 2010. Daily average temperature (Tave, °C), daily maximum temperature (Tmax, °C) and daily average relative humidity (RH, %) were calculated from these hourly records. A temperature-humidity index (THI) was also calculated as proposed by Marai et al. [21] by combining daily average temperature (Tave) in °C with daily average relative humidity (RH) %·0.01:

$$THI = Tave - [(0.31 - 0.31 \cdot RH) \cdot (Tave - 14.4)]$$

Animal material, nucleic acid isolation, DNA amplification and INDELs genotyping.

Genomic DNA from 120 rams pertaining to the experiment carried out by Salces-Ortiz and co-workers in 2013 [16] were used to genotype the INDELs located at the *HSP90AA1* promoter. The polymerase chain reaction was performed from 100ng of genomic DNA using CERTAMP complex amplifications kit chemistry (Biotools, Madrid, Spain) with specific primers (S1 Table). The resulting PCR fragment was purified with ExoSAP-IT (USB Corporation) and sequenced with specific primers (S1 Table). S1 Fig. shows the *HSP90AA1* promoter sequence (DQ983231.1 Ovis aries heat shock protein alpha (HSPCA) gene) in which polymorphisms (SNPs and INDELs) are identified as well as some regulatory motifs, i.e. heat shock element (HSE) and TATA box.

Table 1. Climate parameters in days of blood and sperm samples collection.

blood collection date	Day	AvT	MaT	MiT	Rh	Rhmax	Rhmin	THIavr	THImax	treatment ID
23/03/2010	0	11.6	19.9	3.8	69.0	92.6	37.2	11.87	19.77	Control
	2 to 0	11.9	17.8	6.0	74.8	93.0	46.4	12.09	17.54	
05/07/2010	0	26.8	35.0	16.8	39.4	63.9	19.8	24.47	32.69	July
	2 to 0	25.5	33.1	17.4	44.9	74.1	22.3	23.62	29.88	
03/08/2010	0	24.7	34.4	16.6	49.4	89.3	21.0	23.08	33.74	August 1
	2 to 0	26.1	35.1	16.4	41.3	77.0	17.2	23.95	31.34	
09/08/2010	0	27.3	33.8	22.2	50.0	71.5	28.2	25.30	32.09	August 2
	2 to 0	27.4	35.4	20.3	48.6	76.5	23.23	25.36	32.08	
sperm collection date										
01/03/2010	0	7.8	11.9	4.8	79.5	90.3	58.5	8.22	11.98	
	2 to 0	10.4	16.0	6.4	75.5	91.4	48.0	10.6	15.8	
19/05/2010	0	18.4	26.6	8.1	46.1	82.8	24.1	17.73	25.95	
	2 to 0	16.6	25.7	6.0	50.4	87.2	22.4	16.2	24.0	
29/06/2010	0	23.7	32.8	16.9	58.7	91.6	24.4	22.51	32.32	
	2 to 0	23.7	32.9	15.4	49.7	81.0	19.9	22.2	29.9	
14/07/2010	0	24.9	32.6	17.7	42.2	67.6	20.1	23.02	30.77	
	2 to 0	26.5	34.5	17.9	33.7	58	15.1	24.0	30.3	
27/07/2010	0	26.2	36.4	15.4	45.6	87.8	13.8	24.21	35.57	
	2 to 0	25.6	35.3	16.3	38.8	71.3	15.1	23.5	31.3	
10/08/2010	0	27.5	36.6	19.1	48.6	77.0	24.3	25.41	35.02	
	2 to 0	27.4	35.5	20.3	48.5	75.1	25.3	25.3	32.1	
24/08/2010	0	23.0	31.5	15.9	52.2	86.4	23.6	21.73	30.78	
	2 to 0	24.7	33.3	16.6	48.7	82.6	19.3	23.1	30.4	
05/10/2010	0	14.4	22.7	5.6	67.0	96.3	31.8	14.40	22.60	
	2 to 0	15.1	21.0	8.5	66.4	94.6	39.0	15.0	20.3	

Data are the climate values the day of collection. For blood collection dates, the mean from 2 days before to the day of collection is also presented. Data from: Manzanares (Ciudad Real) Meteorological Station, coordinates 654m–38° 59'47N–03° 22'23W (http://crea.uclm.es/siar). AvT = average temperature (°C); MaT = maximum temperature (°C); MiT = minimum temperature (°C); Rh = average relative humidity (%); Rhmax = maximum relative humidity (%); Rhmin = minimum relative humidity (%); THIavr = THI calculated with the average temperature and relative humidity; THImax = THI calculated with the maximum temperature and the maximum relative humidity; Temperature humidity index (THI) calculated as THI = T-[0.31(1-RH)(T-14.4)], T = temperature in °C; RH = relative humidity/100 [21].

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Linkage disequilibrium analysis

PLINK software [22] (http://pngu.mgh.harvard.edu/purcell/plink/) was used to estimate linkage disequilibrium among the three INDELs here detected and the remaining polymorphisms previously described [17] using r^2, the squared correlation based on genotypic allele counts [23].

Detection of putative transcription factor (TF) binding sites in ovine HSP90AA1 promoter

Putative TF binding sites were *in silico* predicted using Chip Mapper (Multi-genome Analysis of Positions and Patterns of Elements of regulation) [24].

q-PCR reactions and expression analyses

Animals, biological samples, environmental conditions, experimental designs, q-PCR reactions and statistical methods of analysis were the same that those previously described in [16]. Briefly, the experiment consisted on the analysis of *HSP90AA1* RNA content in blood samples

collected from 120 rams in four time points with different climatic conditions. q-PCR assays were performed per triplicate and using the *HSP90AB1* gene as housekeeping to normalize *HSP90AA1* expression results. Statistic methodology to analyze differences in the expression rate of alternative genotypes of some polymorphisms located at the gene promoter was that described by Steibel et al. [25].

Semen samples collection and Sperm DNA fragmentation assay

Animals (61 rams selected from the 120 used in the expression analyses), biological samples, environmental conditions and sperm DNA fragmentation assay (SCSA) data were those used in the work of Ramón and colleagues [15]. Briefly, a total of 7 collections per male were carried out, from March to October as a way to ensure that sperm were exposed to different environmental conditions of temperature and humidity. After collection, sperm samples were incubated at 37°C during 48 hours, and the sperm DNA fragmentation was assessed after collection (0 h) and after 24 and 48 h. Sperm incubation at 37°C has the aim to mimic the environmental circumstances existing at ewe reproductive track. The SCSA assay expressed the extent of DNA denaturation in terms of DNA Fragmentation Index (DFI), which is the ratio of red to total (red plus green) fluorescence intensity, i.e. the level of denatured DNA over the total DNA [26]. The DFI value was calculated for each sperm cell in a sample, and the resulting DFI frequency profile was obtained. Based on the results of the study referred above, only total DNA fragmentation index (tDFI) values were used as dependent variable related to the extent of sperm DNA fragmentation. The tDFI was defined as the percentage of spermatozoa with a DFI value over 25%. Table 2 shows average tDFI values in each incubation stage for the non-heat stress (March to May) and heat stress (June to October) semen collections.

Association analyses among HSP90AA1 genotypes and sperm DNA fragmentation

To assess the effect of heat load on sperm DNA integrity of animals carrying alternative genotypes for the polymorphisms studied, we have examined the relationship among tDFI values and average THI, as suggested in Ramon et al. [15]. Thus, in the present study we compare the degree of sperm DNA fragmentation between genotypes in response to weather conditions from the day 60 previous to semen collection to the day of collection. The aim was to identify those stages of the spermatogenesis in which certain genotypes are more susceptible to heat stress and to identify which polymorphisms play a more important role. For this, a linear mixed-effects model including genotype, sperm incubation time (0h, 24h and 48h), the THI

Table 2. Sperm DNA fragmentation levels (tDFI) at three incubation times for the non-heat stress (NHS) and heat stress (HS) semen collection periods.

Semen collection period	0 h	24 h	48 h
NHS	4.36 ± 0.12	4.64 ± 0.15[a]	6.67 ± 0.77[a]
HS	5.03 ± 0.31	6.75 ± 0.38[b]	12.27 ± 0.81[b]

Data are mean ± standard errors.

tDFI = total DNA fragmentation index (percentage of spermatozoa with a DNA Fragmentation Index (DFI) value over 25%.)

NHS = non-heat stress period (semen collections from March to May)

HS = heat stress period (semen collections from June to October)

[a–b]: different superscript letters within column indicate significant differences between semen collection periods [21].

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(Temperature Humidity Index) and their interactions as fixed effects and male as random effect were conducted. The model was as follow:

$$y_{ijkl} = \mu + IT_j + f(T)_k + G \times f(T)_{lk} + a_i + e_{ijkl}$$

where:

y_{ijkl}: tDFI measure

μ: global mean

IT_j: sperm incubation time (3 levels: 0, 24 and 48 h)

$f(T)_k$: effect of THI for each of the 60 days prior sperm collection

$G \times f(T)_{lk}$: genotype-temperature effect interaction

a_i: male (61 levels)

e_{ijkl}: heterogeneous random residual error ~ N(0, σ $ei2$)

Since it was expected that the effect of temperature on tDFI values was revealed from a threshold, the following function was used to model the temperature effect:

$$f(T)_k = \begin{cases} 0, & \text{if } THI \leq k \\ b(THI - k), & otherwise \end{cases}$$

where, k is the selected threshold. A threshold value of 22 was used, based on the THI heat stress categories reported by Marai et al. [21] and the results previously obtained for this breed [15]. As genotype (G_l), two polymorphisms located at the promoter of the *HSP90AA1* gene were considered and three independent mixed models analyses were conducted based on the genotype included in the model: (i) the SNP G/C-660; (ii) the INDEL-668insC; and (iii) their interaction. Heterogeneous residual variance for the effect of sperm incubation time was also considered. Multiple comparisons among genotypes were conducted for each day, from the day 60 prior to semen collection to date of collection (N = 61), and two scenarios were considered: a Non Heat Stress (NHS) (sperm collections from March and May 2010) and Heat Stress (HS) (sperm collections from June, July, August and October) conditions.

Statistical analysis was performed using the R 3.0.3 statistical language [27]. For multiple comparisons analyses, Bonferroni correction was considered.

Results

Genotype and allele frequencies

Table 3 shows genotypes (a) and allele (b) frequencies of the three INDELs, (-667insC, -668insC and -516insG), and the -660G/C SNP located at the *HSP90AA1* promoter in the 120 rams of Manchega breed used in the expression studies [16] and also for the subset of 61 rams (from these 120) used in the sperm DNA fragmentation assay [15]. For the INDEL -667insC very low frequency of the I$_{-667}$ allele was found. There was only one animal with the ID$_{-667}$ genotype (being I insertion and D deletion) among the 120 rams of the expression analyses. All animals included in the sperm DNA fragmentation assay were homozygous DD$_{-667}$. Therefore, this polymorphism was removed in subsequent analyses. However, the frequency of this polymorphism in the Manchega breed is somewhat greater than that here observed, 0.972 for the D$_{-667}$ allele and 0.027 for the I$_{-667}$ one (data not shown from 48 parent-offspring trios). Also, the INDEL -516insG showed quite extreme frequencies, being the D$_{-516}$ allele the most

Table 3. Genotype and allele frequencies of the three INDELs, -668insC rs397514115.2, -667insC rs397514115.1 and -516insG rs397514268, and the SNP -660G/C rs397514116 located at the *HSP90AA1* promoter in the 120 rams of Manchega breed used in the expression studies and in the 61 rams of them used in sperm DNA fragmentation assays (I = insertion; D = deletion) [15].

Marker	rs#[1]	postion at DQ983231.1 Genbank sequence	Genotype	rams expression (N = 120)	rams expression freq	rams fragmentation (N = 61)	rams fragmentation freq
-668insC	rs397514115.2	g.667_668insC	II	5	0.042	2	0.033
			ID	23	0.192	13	0.213
			DD	92	0.767	46	0.754
-667insC	rs397514115.1	g.666_667insC	II	0	0	0	0
			ID	1	0.008	0	0
			DD	119	0.992	61	1
-660G/C	rs397514116	g.660 G>C	GG	41	0.342	21	0.344
			CG	39	0.325	20	0.328
			CC	40	0.333	20	0.328
-516insG	rs397514268	g.515_516insG	II	2	0.017	1	0.016
			ID	39	0.325	21	0.344
			DD	79	0.658	39	0.639
Marker	rs#[1]	postion at DQ983231.1 Genbank sequence	Genotype	rams expression (2N = 240)	rams expression freq	rams fragmentation (2N = 122)	rams fragmentation freq
-668insC	rs397514115.2	g.667_668insC	I	33	0.138	17	0.139
			D	207	0.863	105	0.861
-667insC	rs397514115.1	g.666_667insC	I	1	0.004	0	0
			D	239	0.996	122	1
-660G/C	rs397514116	g.660 G>C	G	121	0.504	62	0.508
			C	119	0.496	60	0.492
-516insG	rs397514268	g.515_516insG	I	43	0.179	23	0.189
			D	197	0.821	99	0.811

1. rs#= reference SNP ID number

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frequent, 82%, in the samples studied, and was also not included either in the DNA fragmentation assay.

Linkage disequilibrium estimation among 11 polymorphisms of the HSP90AA1 promoter

Table 4 shows the matrix of r^2 values among the 11 polymorphisms (see S1 Fig., for polymorphisms nomenclature) detected at the *HSP90AA1* promoter. Moderate LD values (from 31% to 33%) were found between pairs -516insG_-528A/G, -516insG_-660G/C and -516insG_-704insAA. The -667insC showed a 40% of LD with the SNP -444A/G and the -668insC showed LD values from 19% to 26% with -528A/G, -660G/C and the -704insAA. Among the three INDELs very low r^2 values were found.

Analysis of gene expression for isolate and combined genotypes of INDELs and -660G/C

In the present study, we have examined gene expression rates for alternative genotypes of the INDELs -668insC and -516insG conjunction with the -660G/C polymorphism, since in a previous work [16] animals carrying alternative genotypes of -660G/C temperature dependent differences in *HSP90AA1* gene expression rate were showed. The following contrasts between genotypes within treatments (Control, July, August 1 and August 2) where considered: **a)** contrasts between -668insC genotypes (DD$_{-668}$, ID$_{-668}$ and II$_{-668}$; being I insertion and D, deletion); **b)** contrast between -516insG (DD$_{-516}$, ID$_{-516}$ and II$_{-516}$ genotypes); **c)** contrasts between the

Table 4. Linkage disequilibrium values (r2) among the 11 polymorphisms detected at the *HSP90AA1* promoter.

	-444A/G	-468G/T	-516insG	-522A/G	-524G/T	-528A/G	-601A/C	-660G/C	-667insC	-668insC	-704insAA
-444A/G	1										
-468G/T	0.006	1									
-516insG	0	0.003	1								
-522A/G	0.005	0.003	0.019	1							
-524G/T	0.005	0.901	0.009	0.007	1						
-528A/G	0.045	0.027	0.333	0.011	0.018	1					
-601A/C	0.005	0.619	0.006	0.007	0.689	0.021	1				
-660G/C	0	0.032	0.314	0.038	0.023	0.584	0.025	1			
-667insC	0.398	0.001	0.004	0.001	0.001	0.012	0.001	0.013	1		
-668insC	0	0.003	0.001	0.001	0.002	0.199	0.001	0.251	0.016	1	
-704insAA	0	0.035	0.335	0.041	0.025	0.601	0.027	0.941	0.013	0.264	1

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existent combined genotypes -668insC_-660G/C (DD$_{-668}$CC$_{-660}$; DD$_{-668}$CG$_{-660}$; DD$_{-668}$GG$_{-660}$; ID$_{-668}$CC$_{-660}$; ID$_{-668}$CG$_{-660}$; II$_{-668}$CC$_{-660}$) **d)** contrasts between the existent combined genotypes -660G/C_-516insG (CC$_{-660}$DD$_{-516}$; CC$_{-660}$ID$_{-516}$; CC$_{-660}$II$_{-516}$; CG$_{-660}$DD$_{-516}$; CG$_{-660}$ID$_{-516}$; GG$_{-660}$DD$_{-516}$) and **e)** contrasts between the existent combined genotypes -668insC_-516insG (DD$_{-668}$DD$_{-516}$; ID$_{-668}$DD$_{-516}$; II$_{-668}$DD$_{-516}$; DD$_{-668}$ID$_{-516}$; ID$_{-668}$ID$_{-516}$; DD$_{-668}$II$_{-516}$).

a) Contrasts between alternative genotypes of the -668insC. Fig. 1 shows the results for contrasts comparing cytosine insertion genotypes. Only high statistically significant contrasts were showed. We could observe differences in expression between genotypes only for samples collected in August 1 and August 2, when maximum environmental temperatures exceeded 33°C (Table 1). The homozygote genotype for the insertion (II$_{-668}$) showed much higher expression rates (p<0.0001) than the heterozygote ID$_{-668}$ (FC = 3.07) and the homozygote DD$_{-668}$ (FC = 3.40) for samples collected in August 2 (average temperature = 27.3°C, maximum temperature = 33.8°C and minimum temperature = 22.2°C). For samples collected in August 1 (average temperature = 24.7°C, maximum temperature = 34.4°C and minimum temperature = 16.6°C) lower differences among genotypes than in the previous case were found. Thus, animals carrying the II$_{-668}$ genotype showed higher expression rate (p<0.0001) than those with DD$_{-668}$ (FC = 1.66). In this case also the heterozygote (ID$_{-668}$) had significant (p<0.0001) higher expression levels than the DD$_{-668}$ one (FC = 1.28) but no differences were observed between II$_{-668}$ and ID$_{-668}$ genotypes.

b) Contrasts between alternative genotypes of the -516insG. Fig. 2 shows the results for contrasts comparing guanine insertion genotypes. Only high statistically significant contrasts were showed. We could observe differences of expression between genotypes only for samples collected in July (average temperature = 26.8°C; maximum temperature = 35.0°C; minimum temperature = 16.8°C). The II$_{-516}$ genotype showed higher expression rate than ID$_{-516}$ (FC = 2.49, p<0.0001) and DD$_{-516}$ (FC = 2.35 p<0.001) genotypes. However, as there were only two animals carrying the II$_{-516}$ genotype the standard errors of the estimates were so large.

c) Contrasts of combined genotypes -668insC_-660G/C. Fig. 1 shows high significant contrasts among the existent combined genotypes of both polymorphisms. To facilitate the comparison with results obtained for the isolate -660G/C [16], significant contrasts for genotypes of this polymorphism were also included. In this case, for samples collected under the most extreme heat stress environmental conditions (August 2) gene expression of the combined genotypes is controlled by the genotype of the -668insC. Thus the II$_{-668}$CC$_{-660}$ genotype showed higher expression rates than the DD$_{-668}$CC$_{-660}$ (FC = 3.58) and the ID$_{-668}$CC$_{-660}$ (FC = 3.01). Under climatic conditions existing in August 1, lower effect of the -668insC over gene
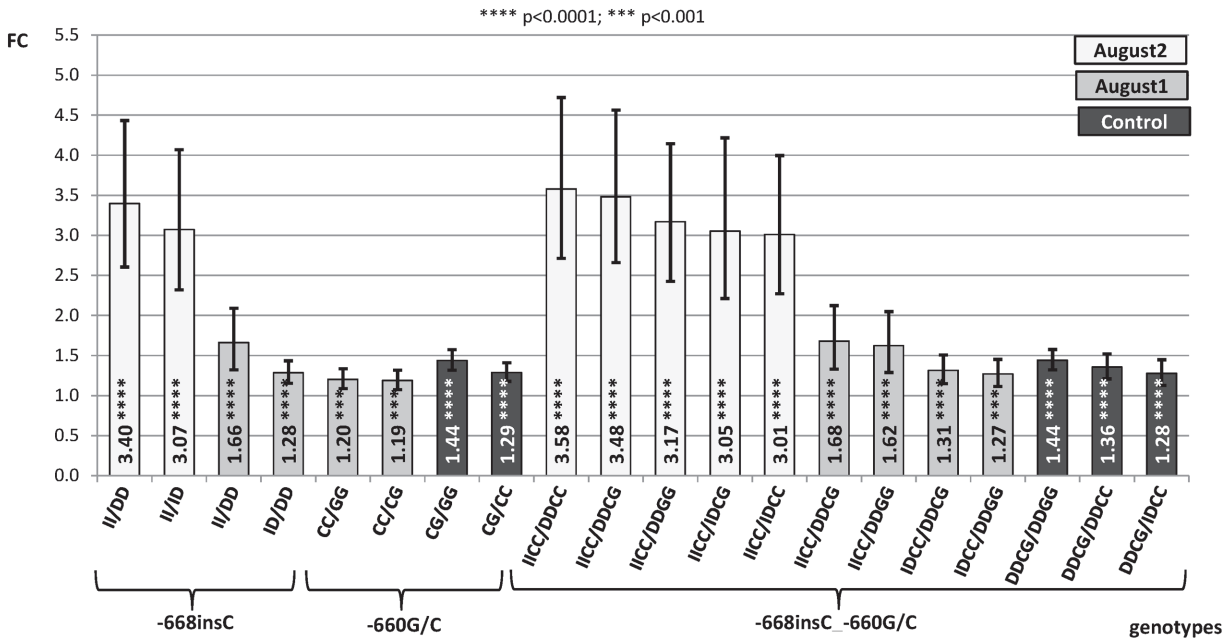
Figure 1. Fold change (FC) for the contrast among alternative genotypes of -668insC, -660G/C and combined genotypes -668insC_-660G/C of the *HSP90AA1* promoter within treatment normalized by *HSP90AB1*. Segments indicate the 95% confidence interval (FC_{up}-FC_{low}). In abscissa the FC, in ordinate genotype contrasts (I = insertion; D = deletion).

expression differences than those observed in August 2 were observed. In this case similar FC (1.6) was found in contrasts between $II_{-668}CC_{-660}/DD_{-668}CG_{-660}$ and $II_{-668}CC_{-660}/DD_{-668}GG_{-660}$. For the contrast $ID_{-668}CC_{-660}/DD_{-668}CG_{-660}$ and $ID_{-668}CC_{-660}/DD_{-668}GG_{-660}$, FC ranged from 1.27 to 1.31. Under mild environmental temperatures (Control) the effect of the -668insC
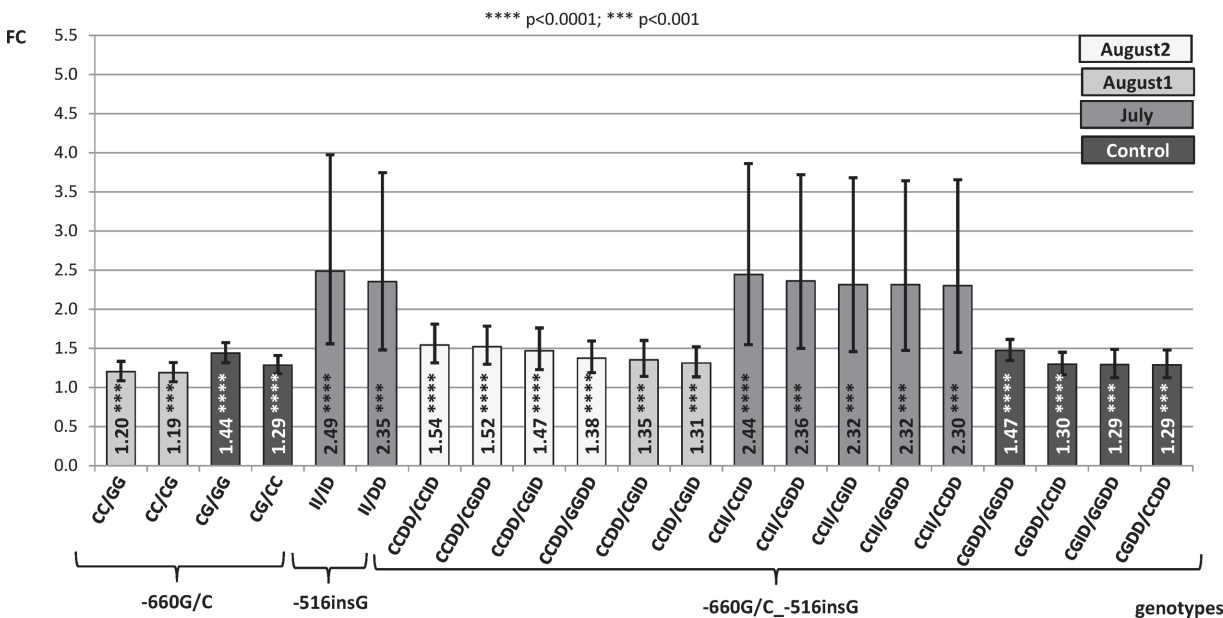

Figure 2. Fold change (FC) for the contrast among alternative genotypes of -660G/C, -516insG and combined genotypes -660G/C_-516insG of the *HSP90AA1* promoter within treatment normalized by *HSP90AB1*. Segments indicate the 95% confidence interval (FC_{up}-FC_{low}). In abscissa the FC, in ordinate genotype contrasts (I = insertion; D = deletion).

genotypes over gene expression differences was lost, and were the genotypes of the SNP -660G/C those revealing expression differences. For Control samples, animals carrying the CG_{-660} genotype showed higher expression levels than those with the GG_{-660} (FC = 1.44) and CC_{-660} (FC = 1.28 to 1.36) independently of the -668insC genotype, as it has been previously observed in [16].

d) Contrasts of combined genotypes -660G/C_-516insG. Fig. 2 shows high significant contrasts among the existent combined genotypes of both polymorphisms. To facilitate the comparison with results obtained for the isolate -660G/C [16], significant contrasts for genotypes of this polymorphism were also included. In significant contrasts from August 1 and 2, except in one case ($CC_{-660}DD_{-516}/CC_{-660}ID_{-516}$), the genotype of the -660G/C transversion seems to be the responsible of differences in the expression rate observed. Thus, animals carrying the CC_{-660} genotype independently of the -516insG one, showed higher expression levels than those with the CG_{-660} (FC from 1.31 to 1.52) and GG_{-660} (FC = 1.38). For the environmental conditions occurred when Control samples were collected, also genotypes of the -660G/C were responsible of differences observed in the expression rate of the gene ($CG_{-660} > GG_{-660}$ FC = 1.3 to 1.4 and $CG_{-660} > CC_{-660}$ FC = 1.3) as in [16].

e) Contrasts of combined genotypes -668insC_-516insG. Fig. 3 shows high significant contrasts among the existent combined genotypes of both polymorphisms. In those contrasts belonging to August 1 and August 2 treatments, the preponderance of the -668insC mutation in the composed genotypes -668insC_-516insG was clear. Thus, homozygous II_{-668} genotype showed higher expression levels than the heterozygous ID_{-668} (FC = 2.81–3.17) and the homozygous DD_{-668} (1.66–3.53), independently of -516insG genotypes. Also, heterozygous $ID_{-668}DD_{-516}$ showed higher expression rate than the $DD_{-668}DD_{-516}$ (FC = 1.32). Results from July are closer to those obtained when considering the -516insG alone. It is important to emphasize
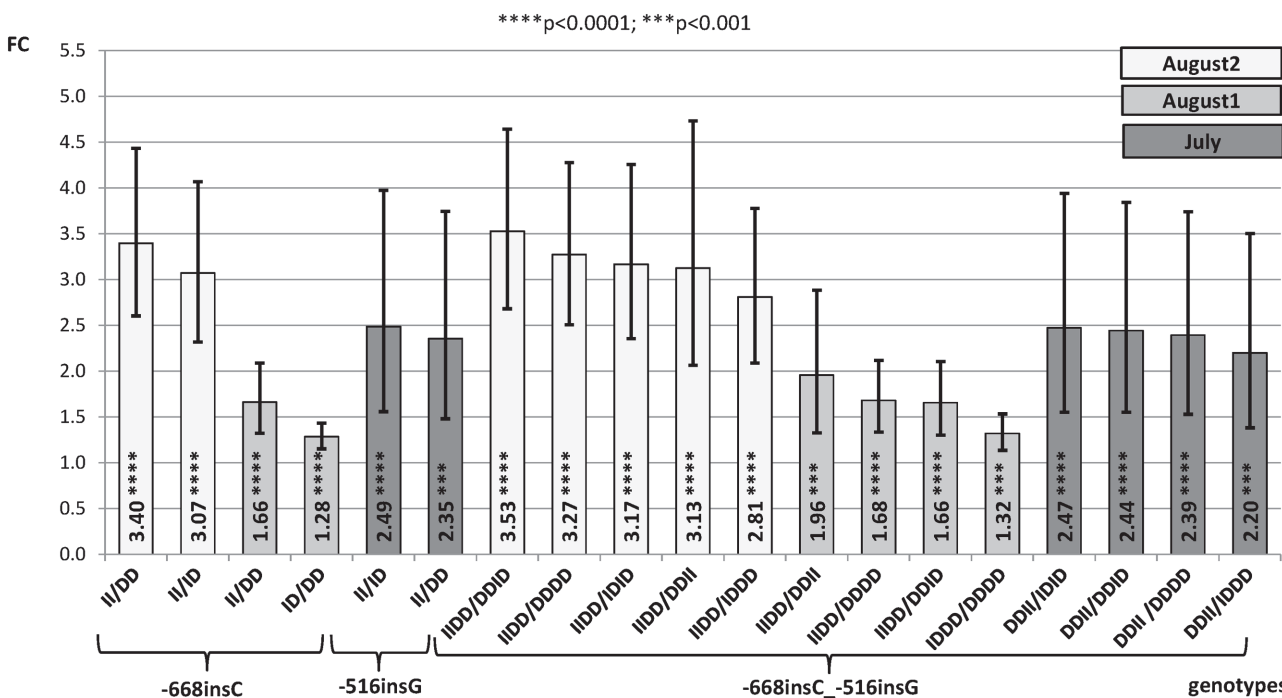

Figure 3. Fold change (FC) for the contrast among alternative genotypes of -668insC, -516insG and combined genotypes -668insC_-516insG of the *HSP90AA1* promoter within treatment normalized by *HSP90AB1*. Segments indicate the 95% confidence interval (FC$_{up}$-FC$_{low}$). In abscissa the FC, in ordinate genotype contrasts (I = insertion; D = deletion).

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that in this case many significant contrasts had high standard errors because a scarce number of animals of a particular genotype in some comparisons. These are the cases in which the II$_{-668}$ and the II$_{-516}$ genotypes were compared, since only five animals have the II$_{-668}$ genotype and only two the II$_{-516}$ one.

Sperm DNA fragmentation as a function of environmental conditions and HSP90AA1 genotypes.

We have observed that as temperature rises, levels of sperm DNA fragmentation also increase with clear differences between non-heat stress semen collections and those conducted under heat stress conditions (Table 2). Fig. 4 shows estimates of the differences of tDFI values between genotypes of the -660G/C, -668insC and the combined genotypes of both mutations depending on THI at each day of the period comprised between days 0 to 60 bsc (before semen collection) for NHS and HS scenarios. Stages of the spermatogenesis process are marked. Bonferroni correction was applied to take into account for multiple tests.

When thermoneutral conditions occurred along the spermatogenesis process, no differences were shown among tDFI values and alternative genotypes of the -660G/C SNP. However,
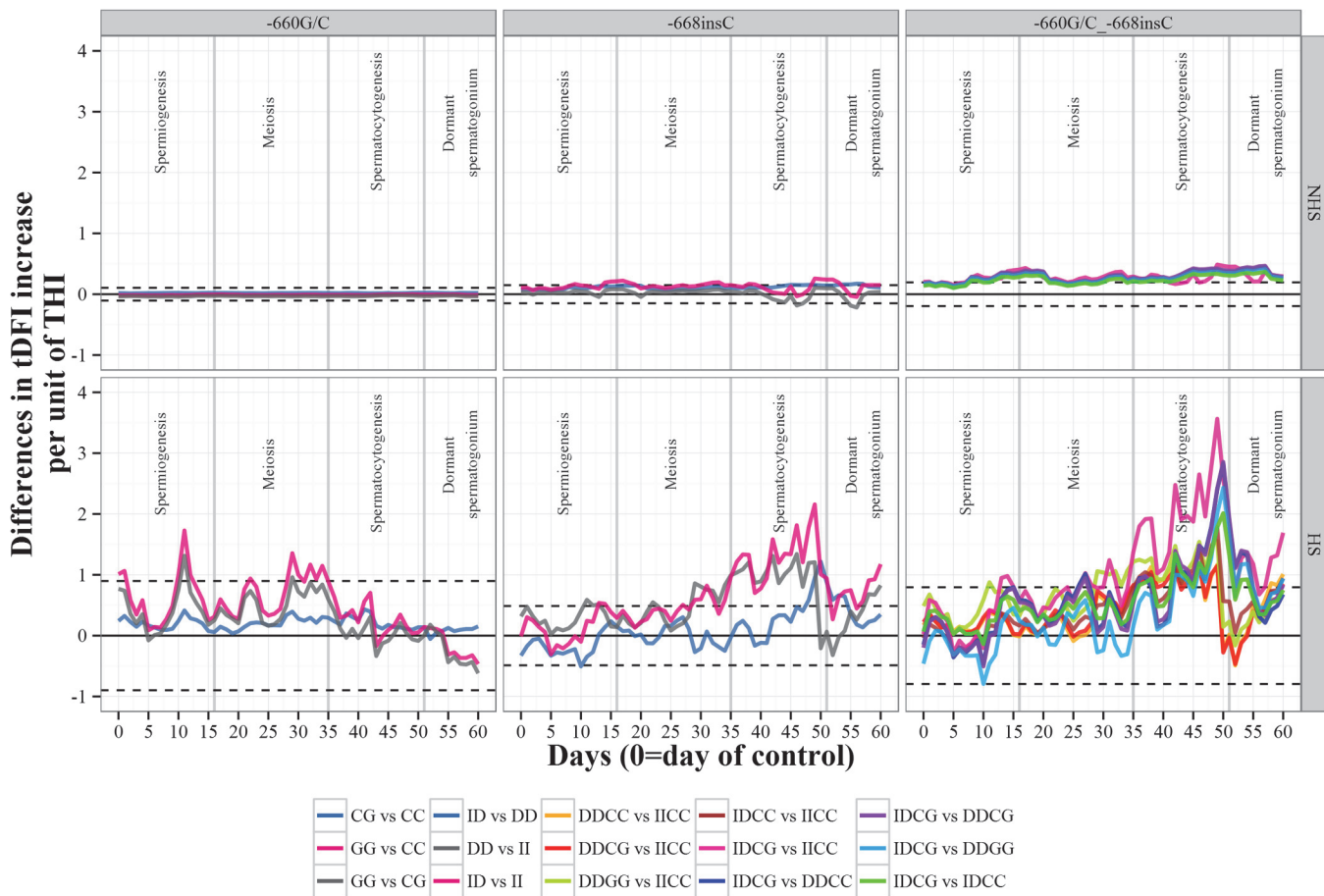

Figure 4. Estimates of the differences of tDFI values between genotypes of the -660G/C, -668insC and the combined genotype of both mutations depending on THI at each day of the period comprised between days 0 to 60 bsc for no heat stress (NHS) and heat stress (HS) collections. Stages of the spermatogenesis process are marked. Contrasts in the first column of the bottom legend apply to -660G/C genotypes. Contrasts in the second column of the bottom legend apply to -668insC genotypes. Contrasts in the third to fifth columns of the bottom legend apply to the combined genotypes.* *For reference values of tDFI under NHS and HS, see Table 2.

significant differences in tDFI values among genotypes were detected when heat stress events occur at some stages of the spermatogenesis. Thus the GG$_{-660}$ genotype showed higher tDFI values than those observed for CC$_{-660}$ when heat stress events occured at periods comprised between days 29 to 34 bsc (1.14 to 1.35 folds per THI unit) and 10 to 12 bsc (1.72 folds). Only for the period 10 to 12 days bsc the GG$_{-660}$ genotype showed higher tDFI values than the CG$_{-660}$ one (1.31 folds). No difference in tDFI values were detected between CC$_{-660}$ and CG$_{-660}$ genotypes.

Under NHS conditions, the ID$_{-668}$ genotype showed greater tDFI values than the II$_{-668}$ at some periods along the spermatogenesis process (0.22 folds for days 15 to 17; 0.19 folds for days 32 to 33; and 0.25 folds for days 49 to 52) but the magnitude of such difference was small. There were two peaks around days 46 and 56 bsc in which the II$_{-668}$ genotype showed higher tDFI values than the DD$_{-668}$ but even with lower magnitude than in the previous case (0.18 to 0.21 folds). When HS conditions took place, more convincing results were obtained. In this way, the ID$_{-668}$ and DD$_{-668}$ genotypes showed very high tDFI values than the II$_{-668}$ (more noticeable for the ID$_{-668}$ genotype than for the DD$_{-668}$ one) in the period comprised between days 37–49 bsc and 29–32 bsc. Thus, for the period 37–49 days bsc ID$_{-668}$ and DD$_{-668}$ genotypes showed tDFI values 1.33 to 2.15 folds and 1.20 to 1.31 folds higher than the II$_{-668}$, respectively. For the period comprised between days 29 to 32 bsc differences among genotypes were smaller than those previously described (0.82 to 0.85 folds for ID$_{-668}$ and DD$_{-668}$ genotypes comparing with II$_{-668}$). These differences decreased along the spermatogenesis process disappearing around day 27 bsc.

When the combined genotypes for the polymorphisms -668insC and -660G/C were considered a mixed pattern but mainly controlled by the INDEL mutation was found. Under NHS conditions the genotype ID$_{-668}$-CG$_{-660}$ was the worst in terms of tDFI values compared with the remaining genotypes in the periods comprised between days 48 to 52 bsc (0.30 to 0.48 folds), 32 to 33 bsc (0.24 to 0.37 folds) and 14 to 17 bsc (0.27 to 0.43 folds). These differences had lower magnitude (0.13 to 0.27 folds) in the central stages of the spermatogenesis process (days 21 to 30). For the HS case (only significant contrasts are shown) much higher magnitude of differences among genotypes were found for the initial period of the spermatogenesis which comprises the period between 36 to 49 days bsc. Again the ID$_{-668}$-CG$_{-660}$ genotype was the one with the highest tDFI values (2.47 to 3.56 folds) when comparing with II$_{-668}$-CC$_{-660}$, DD$_{-668}$-CC$_{-660}$, DD$_{-668}$-GG$_{-660}$ and ID$_{-668}$-CC$_{-660}$ genotypes. Differences among genotypes were decreasing from day 36 bsc to the end of the spermiogenesis stage (1.24 to 1.92 folds). The genotype showing the lowest tDFI values was II$_{-668}$-CC$_{-660}$.

Discussion

The heat shock response is one of the main prosurvival activities of cells. In particular, the sensitivity of mammalian germ cells to environmental heat stress has been extensively studied [28, 29]. Among others, cellular consequences of this stressor are protein miss-folding, DNA damaging, inhibition of DNA repair systems [30], and the inhibition of multiple processes associated with DNA replication [31–33] and the maturation of chromatin [34, 35]. To cope with these effects, cells increase the expression of heat shock proteins (HSPs). This confers a transient protection, leading to a state that is known as thermotolerance, whereby cells become more resistant to various toxic insults, including otherwise lethal temperature elevations. Moreover, HSPs are expressed, though at lower levels, under normal conditions. This observation can be explained by the fact that HSPs are molecular chaperones for protein folding that play a central role in protein homeostasis [36, 37]. There is a constant need for chaperone assistance during *de novo* protein folding and refolding of non native polypeptide chains, as the

stability of cellular proteins is low and aggregation competes with productive folding even at physiological temperatures [38, 39].

Gene expression pattern of the ovine *HSP90AA1* gene has been previously studied [16, 20]. In these works, alternative genotypes of the SNP -660G/C showed differences in gene expression profile under both heat stress and thermo neutral conditions. Thus, animals carrying the CC$_{-660}$ genotype showed higher transcription rates than those with the CG$_{-660}$ (FC = 1.19) and the GG$_{-660}$ (FC = 1.20) ones under heat stress conditions.

In the present study, three INDELs located at the *HSP90AA1* promoter region have been also analyzed regarding their effect over the gene expression rate. Genotypes of the -516insG only showed significant effects over the expression rate of the gene in samples collected in July. These results might involve this mutation with differences in expression under mild heat stress. However, due to the low number of animals carrying alternative genotypes of -516insG this result cannot be confirmed.

The -668insC is very close to the -660G/C transversion, which makes it a good candidate to be implied in the transcriptional regulation of the gene by itself or in a cooperative way with this SNP. The INDEL -668insC is a cytosine insertion located within a region of at least 6 cytosines which made very difficult its exact mapping and genotyping. Alternative genotypes of the -668insC have been here directly associated with differences in the transcription rate of the gene under heat stress environmental conditions but not in thermoneutral ones. Thus, animals carrying the genotype II$_{-668}$ showed higher transcription rates than those with ID$_{-668}$ (FC = 3.07) and DD$_{-668}$ (FC = 3.40) genotypes for samples collected in August 2. However, much lower differences among these genotypes (II$_{-668}$-DD$_{-668}$ FC = 1.66 and ID$_{-668}$-DD$_{-668}$ FC = 1.28) for samples collected in August 1 and no differences for samples collected in July were observed. Despite maximum and average temperatures of these three collection dates are quite similar, some differences should be the clue for the results obtained in each of them. In particular, the minimum temperature in August 2 (22.2°C) was quite higher than in August 1 (16.6°C) and July (16.8°C). The minimum temperature affects one important variable related with the heat stress response, the daily thermal width (TW) which is the difference between the maximum and minimum temperatures occurring along the day. TW values were 18.2°C, 17.8°C and 11.6°C in July, August 1 and August 2 collection dates, respectively. Therefore, it seems that the heat stress response, in terms of over expression of genes involved in this metabolic pathway, rather depends on the daily temperature pattern than on the magnitude of the maximum temperature reached. That is how long is the period of time in which the environmental temperature exceeds a thermoneutral threshold.

To confirm this hypothesis, we examined the climatic conditions in a 3-day period, from 2 days before to the day of sample collection. We observed clearer differences between July samples collection with those carried out in August. For the latter, higher temperature and THI values were observed. Therefore, we confirmed that the magnitude and duration of the stress response is proportional to the dose or severity of the perturbation [40]. These facts would explain the differences in gene expression between genotypes found in the samples collected in July, August 1 and August 2.

The single effects of these two mutations (-660G/C and -668insC) increased when both polymorphisms were considered together (Fig. 1), supporting the hypothesis of a combined action in modulating the gene transcription changes. This was confirmed by a better fit of the model including both polymorphisms (S2 Table), showing that the combination of these two mutations would be able characterize better the response of the animal to thermal stress. The combined II$_{-668}$-CC$_{-660}$ genotype showed the highest expression levels in comparison with the remaining existent genotypes (FC from 1.27 to 3.58) under heat stress conditions. It is important to remark that in the sheep breed here studied (Manchega) these two polymorphisms

showed a LD of 25%. The I_{-668}-C_{-660} haplotype has a frequency of 0.14 while the D_{-668}-C_{-660} and the D_{-668}-G_{-660} have frequencies of 0.37 and 0.49, respectively. The haplotype I_{-668}-G_{-660} does not exist in the ovine species (836 animals from 31 different sheep breeds from different locations of Europe, Africa and Asia have been genotyped, data not shown). Therefore, we cannot completely distinguish the effect of each polymorphism by itself. However, due to the proximity of both mutations, the most likely explanation to the transcriptional upregulation under heat stress is their synergistic effect.

A putative binding site for the Sp1 (specificity protein 1) transcription factor has been predicted for the sequence constituted by these two mutations (S3 Table). Sp1 is a zinc finger transcription factor that binds to GC-rich motifs of many promoters and is involved in many cellular processes, including cell differentiation, cell growth, apoptosis, immune responses, response to DNA damage, and chromatin remodeling. The highest binding affinity of Sp1 was found for the sequence containing the I_{-668} allele and the C_{-660} allele. This affinity decreases for the D_{-668}–C_{-660} haplotype and disappears for the D_{-668}–G_{-660} combination. The high binding affinity of Sp1 for the haplotype I_{-668}–C_{-660} could be the explanation of the higher expression rate observed for the homozygous II_{-668}–CC_{-660} genotype under heat stress conditions than for the remaining ones.

Whatever the mechanism of the gene transcription regulation is, it seems to be clear that genotype x environment dependent transcription rate of the *HSP90AA1* gene observed affects ram's sperm DNA fragmentation in such a way that both events can be linked. Differences in ram's sperm DNA fragmentation have been found by Ramón and colleagues [15] for alternative genotypes of the -660G/C transversion. In the cited work, authors observed that in animals carrying the GG genotype of this SNP sperm DNA fragmentation increases 1.3% per °C (or THI unit) when maximum environmental temperature exceeds 30°C (or average THI > 22) at days 29 to 35 and 7 to 14 before sperm collection (bsc). The same days have been reported by other studies as periods of sperm high sensitivity to heat stress [10, 41]. These periods of time coincide with the meiosis and protamination of sperm cells, respectively, which are critical thermo-sensible stages of the spermatogenesis process. Thus, CG_{-660} and GG_{-660} animals showed significant changes in their sperm DNA fragmentation values depending on environmental variables. However, based in the gene expression rates observed in the present study for the combined genotype -668insC _ -660G/C, and in the fact that both polymorphisms show a moderate LD (25%), the effect of the -660G/C SNP observed by [15] could be modulated by the presence of the -668insC.

In the present work more concluding results, regarding the linkage between gene expression and sperm DNA fragmentation, have been found when analyzing separately tDFI values of sperm samples which have been subjected or not to heat stress along the spermatogenesis process and the combined genotypes of both polymorphisms. When thermoneutral conditions surround the spermatogenesis process (sperm collected in March and May), differences of alternative genotypes of the -668insC and -660G/C mutations analyzed separately were not enough to produce significant differences in sperm DNA fragmentation and only very light when the combined genotypes -668insC _ -660G/C (Fig. 4) were considered (less than 0.5 tDFI units). However when heat stress conditions were present along or at some stages of the spermatogenesis process (sperm collected in June, July, August and October) different results were observed when considering the -668insC and -660G/C genotypes individually or combined, not only in the magnitude of the differences in tDFI values observed among genotypes but also in the spermatogenesis stage where heat stress has greater effect over sperm DNA fragmentation measured after 48h of 37°C heating after ejaculate collection.

Our results confirmed that both polymorphisms are involved in the effect that climatic conditions has over sperm DNA fragmentation and that previous results [15] associated to the

-660G/C were masking the effect of the INDEL -668insC, which resulted to be more important. Fig. 4 shows similar peaks at the spermiogenesis and meiosis stages to those observed for the -660G/C alone. However, the critical stage in terms of heat stress effect over sperm DNA fragmentation differences moved to the spermatocytogenesis stage in where the maximum tDFI differences between genotypes were observed. These differences ranged from 1.27 to 1.32 folds for the $DD_{-668}GG_{-660}$ vs. $II_{-668}CC_{-660}$, and from 1.92 to 3.56 folds for the $ID_{-668}CG_{-660}$ vs. $II_{-668}CC_{-660}$. Differences in the gene expression rate for these same genotypes were also high (FC = 1.6 to 3.1) when heat stress conditions (August 2 and August 1) were present. Therefore, it seems reasonable to consider that both events are correlated, but in what way?

It is well known that there are differences in the genes transcription rate in the stages (cell types) of the spermatogenesis process [41–43] and also in the heat stress sensitivity of the different cell types involved [10, 41, 44]. In the spermatocytogenesis stage (mitosis), spermatogonia and primary spermatocytes have high transcription levels as occurs in other undifferentiated cells. However these levels decay in meiotic cells (secondary spermatocytes and spermatids) and in mature spermatozoa. Moreover, cell-specific genes are transcribed at each stage of the spermatogenesis process [42]. In rats, the expression levels of the HSP90AA1 gene decrease drastically (80%) during the early phases of the spermatogenesis reaching undetectable levels in the more mature germ cells [42] as it has been observed for our group in sheep spermatozoa (data not shown). During germ cell development, different spermatogenic cell types showed remarkable variation in their susceptibility to heat stress being spermatogonia and spermatozoa the most thermotolerant cells while pachytene spermatocytes and early spermatids are more susceptible to heat [3, 7].

Taking into account this background, we can make some hypothesis around the results here obtained regarding the expression levels observed for the HSP90AA1 gene in animals carrying alternative combined genotypes of the -668insC_ -660G/C mutations and the variation of the tDFI values observed in the spermatozoa of these same animals depending on heat stress events occurring along the spermatogenesis process. The highest differences in tDFI values among -668insC_ -660G/C combined genotypes were observed when the THI threshold was exceeded during the spermatocytogenesis stage, independently from heat stress events occurring in posterior phases of the spermatogenesis process. Heat stress at this stage induces the expression of the HSP90AA1 gene. Thus unfavorable genotypes in terms of gene expression induction ($ID_{-668}GC_{-660}$, $DD_{-668}GG_{-660}$) do not produce enough mRNA (mRNAs are stored as messenger ribonucleoprotein particles [45]) and Hsp90α protein to cope with future thermal stress which might occur in posterior stages in which transcriptional activity is reduced and cell types and molecular processes are more sensible to heat (spermatocytes in pachytene and spermatids protamination).When THI threshold was exceeded in the meiosis and spermiogenesis stages differences in tDFI values of alternative combined genotypes of -668insC_ -660G/C are much lower than those observed in the previous case described maybe due to the limited transcriptional activity of the cellular types here involved. Two peaks of higher differences corresponding to meiosis and protamination could indicate the importance of past (selective translation of stored mRNAs [46]) and present (limited) expression rates of the HSP90AA1 gene to protect the meiotic process and produce an optimal exchange of histones by protamines [46, 47] to achieve an optimal spermatozoa DNA packaging.

Therefore, optimal expression rates of favorable genotypes of the HSP90AA1 gene induced by heat stress events seem to be related with a higher ability of mature spermatozoa to cope with the effects that high temperatures exert over their DNA fragmentation when they are subjected to 37°C for 48h. This ability must consist essentially in a better packaging of the sperm DNA (efficient protamination) during the spermiogenesis process which would be favored by higher amounts of Hsp90α, translated at this moment or stored in the past. In bulls, the DNA

fragmentation index (DFI), has been positively correlated with the percentage of spermatozoa that showed low protamine content [Fortes et al, 2014]. However, other roles of the Hsp90α related with the cellular defense against other sources of stress (i.e. oxidative stress) and proteostasis maintenance [48] must not be discarded to preserve spermatozoa DNA from injuries.

Results here obtained lead us to question if heat stress events occurring at initial stages of the spermatogenesis process would be selectively advantageous to protect cell types of subsequent stages, which have worse heat stress response in terms of transcription ability, from injuries caused by heat or other sources of stress. Relative to this idea, it is important to remark that sheep is a short day breeder whose favorable reproductive period begins when the days shorten (fewer hours of light). This period comes after the hottest months, and so, we could expect that those animals with a favorable genotype in terms of heat resistance were more fertile.

Future functional *in vitro* studies, can contribute to elucidate which polymorphism(s) and transcription factor(s) are involved in the expression differences observed in this gene as response to environmental conditions. Also the methylation pattern of the *HSP90AA1* promoter would provide information about other possible mechanism for the regulation of the gene expression. Finally, association studies among sperm DNA fragmentation and *HSP90AA1* genotypes with ram's fertility will contribute to determine the involvement of this gene in ram's reproductive cells thermo sensibility and its consequences over their reproductive efficiency.

Supporting Information

S1 Fig. *HSP90AA1* promoter region containing the polymorphism positions. In dark grey the heat shock element (HSE) binding heat shock factor 1 (HSF1). In light grey the TATA box sequence.
Polymorphisms identifications are the following: [1]-704insAA; [2]-668insC (rs397514115.2); [3]-667insC (rs397514115.1); [4]-660G/C (rs397514116); [5]-601A/C (rs397514117); [6]-528A/G (rs397514269); [7]-524G/T (rs397514270); [8]-522A/G (rs397514271); [9]-516insG (rs397514268); [10]-468G/T (rs397514272); [11]-444A/G (rs397514273).
(DOCX)

S1 Table. List of primers used to amplify and/or sequence the *HSP90AA1* gene polymorphisms.
(DOC)

S2 Table. Goodness of fit criteria for models used to analyze expression data.
(DOC)

S3 Table. Putative transcription factors predicted for the -668insC and -660G/C polymorphisms by Chip Mapper [24].
(DOCX)

Acknowledgments

Author Contributions

Conceived and designed the experiments: MR MMS. Performed the experiments: JSO MR CG MDPG JJG OGA AMM MMS. Analyzed the data: MR MMS JSO. Contributed reagents/

materials/analysis tools: MR MMS JJG JHC MDPG. Wrote the paper: MR JSO CG JHC MMS. Designed the software used in analysis: MR.

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