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# Maternal Exposure to High Temperatures Disrupts OCT4 mRNA Expression of Rabbit Pre-Implantation Embryos and Endometrial Tissue

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**Contents**

We examined the effect of prolonged high heat stress on reproductive performance and its relationship with gene expression in pre-implantation embryos and endometrial tissue. In experiment 1, primiparous rabbit does were divided into two environments: control does (maintained between 14 and 22°C) and heat-treated does housed in a climatic chamber (maintained between 25 and 35°C). Females were reproducing, and the litter size and live born kits were assessed at 2nd and 3rd partum. In heat-treated does, lower litter size (9.7 ± 0.48 and 11.4 ± 0.50) and fewer live born kits (7.2 ± 0.55 and 10.2 ± 0.57) were observed, although similar ovulation rates and numbers of pre-implantation embryos were noted. In experiment 2, after 3rd partum multiparous non-lactating does from each experimental group were used to obtain pre-implantation embryos and endometrial tissue. mRNA transcripts from *OCT-4*, *VEGF*, *erbB3*, *Ifn-γ*, *HSP70* and *HSP90* were analysed by real-time qPCR. Higher values of *OCT-4* expression were observed in embryos and endometrial tissue in females reproduced under heat conditions. Moreover, elevated temperatures have been shown to up-regulate *VEGF* in embryos and down-regulate *Ifn-γ* in endometrial tissue. The findings suggest a deleterious temperature effect on litter size and live born kits as a consequence of variation in gene expression pattern of the pre-implantational embryo and the endometrium associated with proliferation and differentiation and probably with implantation and uterine and foetal development during gestation.

## Introduction

The effect of high temperatures on rabbit doe performance has been studied by experiments carried out in summer (Méndez et al. 1986; Marai et al. 2002), creating the desired heat conditions in climatic chambers (Fernández-Carmona et al. 1995, 2003) or artificially under *in vitro* culture of oocytes and embryos (Alliston et al. 1995; Makarevich et al. 2007). Heat stress causes alterations in the biological processes and functions (Hansen 2009).

Among heat effects in females, the main alteration is a feed intake reduction. Therefore, some metabolites would not be available for metabolism disturbing enzymatic reactions, hormones and blood composition (Wittroff et al. 1998; Marai et al. 2002). Heat stress thus alters several aspects of reproductive physiology, such as blood flow and steroidogenesis (Roman-Ponce et al. 1978; Trout et al. 1998; Rivera and Hansen 2001), which manifests in fertility alterations. It is widely accepted that the traits mainly affected by heat stress in females are puberty, ovulation rate, conception rate, implantation and embryonic development and viable embryos per doe, litter size, litter weight, milk-production and pre- and post-weaning mortality (Marai et al. 2002).

Heat stress during pregnancy results in changes in endocrine dynamics of the maternal unit due to disturbances in the activity of the hypothalamo-pituitary-adrenocortical axis (HPA) and sympatho-adrenal medullary axis (Minton 1994). Modification of these glands causes changes in endocrine profiles of hormones such as cortisol and corticosterone, epinephrine, triiodothyronine and thyroxine, oestrone sulphate, progesterone, aldosterone, glucocorticoids and catecholamines (Thompson et al. 1963; Collier et al. 1982; Magbud et al. 1982; Minton 1994; Kumar et al. 2011). This hormonal dysfunction has been reported to cause lower birth weights, reduced conceptus function, delay in foetal growth, miscarriage, stillbirths and consequently reduced fertility in females (Collier et al. 1982; Bell et al. 1989; Malayer et al. 1990; Kumar et al. 2011). In addition, an elevation of body temperature affects uterine environment by decreasing blood flow, responsible for providing nutrients, oxygen and water to the developing embryo, ovary, cervix and oviduct (Senger et al. 1967; Bazer et al. 1969; Roman-Ponce et al. 1978; Lubin and Wolfenson 1996; García-Ispierto et al. 2006). These maternal body alterations due to heat stress have been shown to cause important disturbances at different stages of embryonic development, such as cleavage and blastocyst formation (Wolfenson and Blum 1988; Rivera and Hansen 2001; Matsuzuka et al. 2005). Reduced embryo development is not just a consequence of body temperature, but also of the interaction between embryo and uterine environment (Ozawa et al. 2002; Matsuzuka et al. 2005).

The current study examines the effect of prolonged high heat stress, as occurs in summer in the Mediterranean area, on reproductive performance and its relationship with gene expression in pre-implantation embryos and endometrial tissue.

## Materials and Methods

All chemicals in this study were purchased from Sigma-Aldrich Química S.A. (Madrid, Spain) unless stated otherwise.

### Animals

Rabbit does belonging to Line V from the ICTA at Polytechnic University of Valencia (Spain) were used to obtain embryos. The V line is selected for litter size at weaning (Estany et al. 1989). The Ethics and Animal

Welfare Committee of the Universidad Politécnica de Valencia approved this study. All animals were handled according to the principles of animal care published by Spanish Royal Decree 1201/2005 (BOE, 2005; BOE = Official Spanish State Gazette).

In this study, rabbit does were divided into two groups: control (14–22°C) and heat-treated (25–35°C) does. In the heat-treated group, does were housed in a climatic chamber (13 × 4.7 × 3.1 m) of 175m$^3$ of air (García-Diego et al. 2011). In this chamber, animals were maintained with sinusoidal curve in a range of 25 to 35°C and humidity range from 11.8% to 63.8%, reaching the minimum at 12 p.m. and maximum at 12 a.m. (García-Diego et al. 2011). In the control group, does were housed in a conventional room with temperatures varying between 14 and 22°C and a humidity range from 28.8% to 65.2%. The actual summer and winter conditions of the Spanish Mediterranean area were reproduced in both experimental groups.

## Experimental design

In this study, two sequential experiments were conducted. In experiment 1, litter size and number of kits born alive from heat-treated females were assessed and compared with the control group. In experiment 2, mRNA expression of *OCT-4*, *VEGF*, *erbB3*, *Ifn-γ*, *HSP70* and *HSP90* on pre-implantation embryos and endometrial tissue for heat-treated females was assessed and compared with control group samples.

### *Experiment 1. Reproductive performance under high temperature conditions*

In this experiment, a total of 22 heat-treated does and 20 control does were used. Heat-treated females were acclimated to climatic chamber during 1st partum. During 2nd and 3rd partum, the female's reproductive behaviour was reported. Both groups of does were artificially inseminated when receptive (identified by red vulvar lips) with pooled sperm, using a standard curved plastic pipette (Imporvet, S.A., Barcelona, Spain). At the same time, each female was given an intramuscular injection of 1 μg of buserelin acetate to induce ovulation. Litter size and number of kits born alive during 2nd and 3rd partum were reported.

### *Experiment 2*

In this experiment, after the 3rd partum, 11 multiparous non-lactating does from each group were artificially inseminated as previously described, 48 h after synchronization with 25 UI of PMSG (Foligon®; Intervet, Boxmeer, Holland). Then, females were slaughtered 6 days post-artificial insemination. Reproductive tracts were dissected, and ovulation rates were recorded. Embryos were recovered by flushing of uterine horns with 20 ml of DPBS (Dulbecco's phosphate-buffered saline) supplemented with 0.1% of BSA. The number of pre-implantational embryos recovered for each female was noted. In addition, endometrium samples were obtained from each female by gently scraping. Samples were stored at -20°C in RNA later solution (Qiagen Iberia SL, Madrid, Spain) until RNA extraction.

## Total RNA extraction and cDNA synthesis

Total RNA was isolated from 13 different pools consisting of six to ten pre-implantational embryos (6 for control group and 7 for heat-treated group). The gene expression for endometrial tissue was performed in 17 females (8 for control group and 9 for heat-treated group). Total RNA was isolated by traditional phenol/chloroform method using sonication in reagent Trizol (Invitrogen S.A, Barcelona, Spain). To prevent DNA contamination, one deoxyribonuclease treatment step (gDNA Wipeout Buffer, Qiagen Iberia SL) was performed from total RNA. Afterwards, 1μg of total RNA isolated from pre-implantational embryos and endometrial tissue was reverse transcribed into cDNA in a total volume of 20 μl using QuantiTect Reverse Transcription Kit (Qiagen Iberia SL), following the manufacturer's instructions.

## Primers design

Two genes, histone (*H2afz*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), were selected as reference genes in the quantitative RT reverse transcriptase-polymerase chain reaction (qRT-PCR) in accordance with Llobat et al. (2011). BESTKEEPER (version 1) software was used to analyse the stability of both reference genes (Pfaffl et al. 2004). Furthermore, gene specific primers were assayed to evaluate *OCT-4*, vascular endothelial growth factor (*VEGF*), epidermal growth factor receptor-3 (*erbB3*), interferon-gamma (*Ifn-γ*), and heat shock protein 70 and 90 (*HSP70*, *HSP90*) expression (Table 1). Specific primers were designed exon to exon for *HSP70* and *HSP90* gene expression (Table 1). All primers have efficiency between 86 and 110 and a correlation between 0.986 and 0.999 in both tissues. Genomic DNA and total RNA (pool from embryos) samples were run to check possible amplification. To avoid DNA contamination, DNA and RNA samples were run to check possible amplification. The products of real-time PCR were confirmed by ethidium bromide-stained 2% agarose gel electrophoresis in 1× TBE buffer.

## SYBR Green assay (qPCR)

The mRNA expression was analysed by real-time PCR in an Applied Biosystems 7500 (Applied Biosystems, Foster City, CA, USA). PCR was performed from diluted (1 : 10) cDNA template. Five microlitre of each sample was used in each reaction in a final volume of 20 μl with 10 μl of 2× SYBR Green Master Mix (Thermo Scientific, Fisher Scientific, Madrid, Spain), 250 nM of forward and reverse primers (list of real-time PCR primers is shown in Table 1). The PCR protocol included an initial step of 50°C (2 min), followed by 95°C (10 min), 42 cycles of 95°C (1 s) and 60°C (1 min). After real-time PCR, a melting curve analysis was performed by slowly increasing the temperature from 65°C to 95°C, with continuous recording of changes in fluorescent emission intensity. Serial dilutions of cDNA

Table 1. Primer sequence, accession number, amplicon size obtained, efficiency, correlation and reference where indicated, of genes analysed and housekeeping genes used (*OCT-4*, transcript of POU5F1 gene; *VEGF*, as vascular endothelial growth factor; *Inf-γ*, as interferon-gamma; *erbB3*, as epidermal growth factor receptor 3; *HSP70*, as heat shock protein 70; *HSP90*, as heat shock protein 90; histone (*H2afz*) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as housekeeping gene)

| Gene | Accession number | Sequence | Fragment size (pb) | Reference |
|---|---|---|---|---|
| *OCT 4* | NM_001099957 | For – 5′CGAGTGAGAGGCAACTTGG<br>Rev – CGGTTACAGAACCACACACG | 125 | Mamo et al. (2008) |
| *VEGF* | AY196796 | For – 5′CTACCTCCACCATGCCAAGT<br>Rev – 5′CACACTCCAGGCTTTCATCA | 236 | Saenz-de-Juano et al. (2011) |
| *Inf-γ* | NM_001081991 | For – 5′GTCTGCATTCTAGCCACTG<br>Rev – 5′ATTCAGGGGCAGTCACAGTT | 151 | Llobat et al. (2011) |
| *erbB3* | AF333179 | For – 5′GTCACATGGACACGATCGAC<br>Rev – 5′AAGCAGTGGCCGTTACACT | 191 | Saenz-de-Juano et al. (2011) |
| *HSP70* | 100354037 | For – 5′GGAAGCACAAGAAGGACCTG<br>Rev – 5′CCTGGTGATGGACGTGTAGA | 158 | |
| *HSP90* | ENSOCUT00000013430 | For – 5′AGAGGTTCCTGCACCATGAG<br>Rev – 5′TTCCCGCTCCATAAAAAGTG | 177 | |
| *H2afz* | AF030235 | For – 5′AGAGCCGGCTGCCAGTTCC<br>Rev – 5′CAGTCGCGCCCACACGTCC | 85 | Mamo et al. (2008) |
| *GAPDH* | L23961 | For – 5′GCCGCTTCTTCTCGTGCAG<br>Rev – 5′ATGGATCATTGATGGCGACAACAT | 144 | Navarrete-Santos et al. (2004) |

pool made from several samples were run in triplicate to asses PCR efficiency and decide which dilutions to use for unknown samples. A $\Delta\Delta C_t$ method adjusted for PCR efficiency was used (Weltzien et al. 2005), employing as normalization factor the geometric average of *H2afz* and *GAPDH*. Target and reference genes in unknown samples were run in duplicate. Relative expression of cDNA pool from all samples was used as a calibrator. Non-template control (cDNA was replaced by water) for each primer pair was run in all plates.

### Statistical analysis

Total litter size, number of kits born alive, ovulation rate and 6-day-old embryos recovered were analysed by one-way ANOVA, using the general linear models (GLM) procedure of STATGRAPHICS PLUS 5.1 (STSC Inc., Rockville, MD, USA). For mRNA expression, differences in mRNA expression among different groups in each comparison were also analysed by one-way ANOVA, using the GLM procedure. For *HSP90* and *HSP70* in endometrial tissue, a Neperian logarithmic transformation was carried out before analysis for data normalization. Differences of $p < 0.05$ were considered significant.

## Results

### Experiment 1

Litter size and number of kits born alive reported to be different between heat-treated and control group females, being lower in does submitted to heat treatment than in control group (Table 2).

### Experiment 2

*Ovulation rate and pre-implantation embryo recovery*

Ovulation rate did not show significant differences between heat-treated and control group females (Table 3). In the same line, the number of pre-implantation embryos recovered from heat-treated and control group females was also similar (Table 3).

Table 2. Litter size, number of kits born alive for heat-treated and control group females (mean ± SEM)

| Experimental group | n | Litter size | Live born kits |
|---|---|---|---|
| Heat treatment | 22 | $9.7 \pm 0.48^b$ | $7.2 \pm 0.55^b$ |
| Control | 20 | $11.4 \pm 0.50^a$ | $10.2 \pm 0.57^a$ |

Different superscripts in the same column represent a significant difference ($p < 0.05$).
n, number of females used.

Table 3. Ovulation rate and pre-implantation embryos recovered for heat-treated and control group females (mean ± SEM)

| Experimental group | n | Ovulation rate | Recovered embryos |
|---|---|---|---|
| Heat treatment | 11 | 17.8 ± 1.15 | 12.5 ± 1.47 |
| Control | 11 | 16.6 ± 1.70 | 11.8 ± 2.18 |

n, number of females used.

*mRNA expression*

The expression level of *HSP90* in pre-implantation embryos was too low to obtain a correct efficiency curve to work with, so the analysis of mRNA expression of this gene was dismissed only for embryos. BestKeeper showed that both *H2afz* and *GAPDH* are stable in pre-implantation embryos and endometrial tissue (0.60 vs 0.53 and 0.65 vs 0.53, for pre-implantation embryos and endometrial tissue, respectively). The geometric average of both was therefore used as reference.

Differences in mRNA transcript abundance between pre-implantation embryos and endometrial tissue in both conditions are shown in Figs 1 and 2. In pre-implantational embryos, the level of expression of *OCT-4* and *VEGF* was higher for heat-stressed females ($1.27 \pm 0.06$ vs $0.89 \pm 0.09$ and $2.50 \pm 0.25$ vs $1.12 \pm 0.35$, for heat-stressed and control females, respectively). No differences were detected for *Inf-γ*, *erbB3* and *HSP70*.

According to previous results in pre-implantational embryos, in endometrial tissue, *OCT-4* expression also

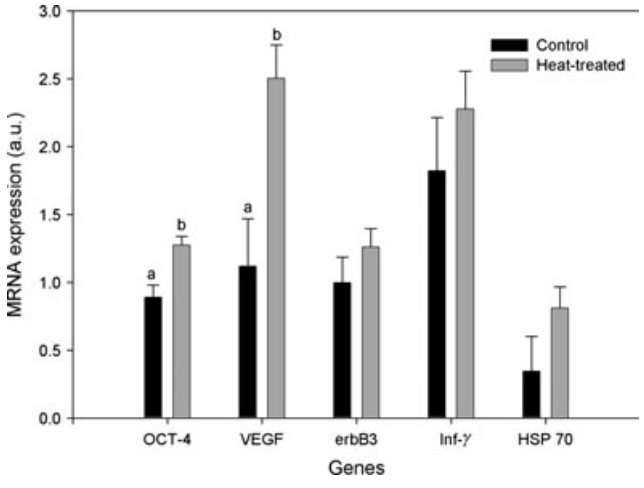


Fig. 1. Relative expression of transcription factor *OCT-4*, vascular endothelial growth factor (*VEGF*), epidermal growth factor receptor 3 (*erbB3*), *Inf-γ*, interferon-gamma and heat shock protein 70 (*HSP70*) for pre-implantation embryo of control and heat-treated females. Relative abundance values are expressed by the mean value ± SEM of the replicates of each group. a.u.: arbitrary units. Bars with different letters are significantly different ($p < 0.05$)

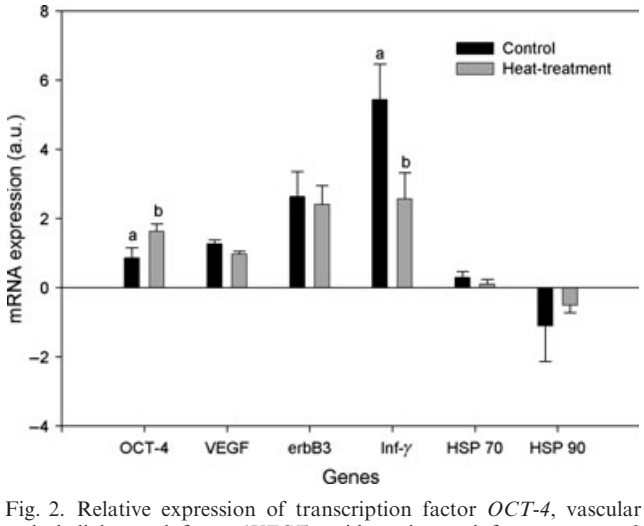


Fig. 2. Relative expression of transcription factor *OCT-4*, vascular endothelial growth factor (*VEGF*), epidermal growth factor receptor 3 (*erbB3*), *Inf-γ*, interferon-gamma, heat shock protein 70 (*HSP70*), heat shock protein 90 (*HSP 90*) for endometrial tissue of control and heat-treated females. Relative abundance values are expressed by the mean value ± SEM of the replicates of each group. a.u.: arbitrary units. Bars with different letters are significantly different ($p < 0.05$)

showed higher values for heat-treated than for control females (1.63 ± 0.21 vs 0.86 ± 0.29). On the other hand, *Ifn-γ* reported lower expression in heat-treated than in control females (5.43 ± 1.03 vs 2.56 ± 0.76). The level of expression of *HSP90*, *VEGF*, *erbB3* and *HSP70* was similar between both groups of females in this tissue.

## Discussion

It is widely known that high temperatures lead to a decrease in fertility and prolificacy (Wolfenson et al. 2000; Marai et al. 2002; De Rensis and Scaramuzzi 2003; Hansen 2009). The current study indicates that the adverse effect of high temperatures on reproductive performance is associated with variation in the gene expression of embryos and endometrial tissue before the implantation, but not with a reduced ovulation rate, fecundation rate or proportion of embryos developing to the late blastocyst stage. The findings of this study indicate similar numbers of recovery pre-implantation embryos, as previously reported (Edwards et al. 1968; Omtvedt et al. 1971). Nevertheless, several studies in rabbit and other mammalian species have shown that high temperature causes a reduction in the litter size (Marai et al. 2002; Fernández-Carmona et al. 2003). It has also been demonstrated that reduction in litter size is approxiamtely 14% (current study) and in line with previous reports (Ayyat and Marai 1998). This decline seems to be associated with the implantation process, as studies pre- and post-implantation in heat-stressed females have shown a drop in the number of implantations and viable embryos after this process (Alliston et al. 1965; Omtvedt et al. 1971; Wolfenson and Blum 1988). Omtvedt et al. (1971) established that heat stress still has a great effect on foetal mortality during late gestation in gilts. However, Edwards et al. (1968) reported that embryos are less susceptible to heat stress after implantation. Nevertheless, studies carried out with embryos under *in vitro* conditions showed less development to the blastocyst stage when embryos were exposed to heat stress at 1 cell stage and 2 cell stage, but when *in vivo* early development was assessed, the same number of implantations was reported (Alliston et al. 1965; Rivera and Hansen 2001; Matsuzuka et al. 2005).

Heat stress has been documented to induce alterations in the transcriptional levels of genes involved in cell growth, cell cycle and programmed cell death (Gasca et al. 2008). Our results provided evidence of an altered *OCT-4* expression in both embryonic and endometrial tissue in females reproducing under high temperature, as previously reported (Gendelman and Roth 2011). *OCT-4* is regarded as a key regulator of the pluripotency maintenance system (Boiani and Schöler 2005). The main function of this transcriptional factor is to activate or repress several target genes involved in many cases in cell differentiation and early embryonic development (Smith et al. 2007). *In vivo* develop of rabbit embryos shown a pattern of down-regulation in *OCT-4* transcripts in 5- and 6-day-old blastocysts compared to 4-day-old blastocysts, and it has been suggested as an intrinsic embryo mechanism to enable successful implantation (Saenz-de-Juano et al. 2010). Therefore, up-regulation in *OCT-4* transcripts in embryos observed in our study supports the assumption that these embryos have a developmental delay. The altered expression of *OCT-4* in pre-implantational embryo is associated with embryos of lower quality (Gendelman and Roth 2011). *OCT-4* mRNA expression has been detected in human endometrial stromal (Matthai et al. 2006; Bentz et al. 2011), although to our best knowledge, expression in endometrial tissue has not been previously reported in rabbit. *OCT-4* expression strongly suggests the existence of endometrial stem cells, probably in stromal, that must be able to differentiate into mesenchymal tissues, given the remarkable plasticity, regenerative capacity and growth of endometrium (Park et al. 2011). Furthermore, differ-

ential regulation of *OCT-4* could reflect uterus perturbations and might explain, in part, the decrease in litter size observed in heat-stressed does (current study) and in previous reports (Marai et al. 2002; Fernández-Carmona et al. 2003). In addition, *Inf-γ* in endometrial tissue and *VEGF* in pre-implantation embryos also showed altered regulation. They are related to implantation, as *VEGF* is involved in the connection of foetal to maternal blood supplies (Cullinan-Bove and Koos 1993), and *Inf-γ* is related to pregnancy recognition (Godornes et al. 2007). In fact, previous studies have reported that *OCT-4* affects the expression of *VEGF* and inhibits human chorionic gonadotrophin expression (Liu et al. 1997; Yamamoto et al. 1998), which could explain the related alterations, as suggested by Llobat et al. (2011).

The expression of *HSP70* in embryos and endometrial tissue and *HSP90* in endometrium did not differ among heat-treated and control females. HSPs are synthesized in response to a wide variety of cellular injuries that are induced by changes in temperature as well as other stress conditions, such as the presence of free oxygen radicals, infections, heavy metals, ethanol and ischaemia (Lindquist 1986; Welch 1992). Increased HSP70 expression is associated with increased ability of the stressed cells to protect themselves from cell death (Mosser et al. 1997) by interacting with apoptotic mechanisms (Buzzard et al. 1998; Li et al. 2000; Parcellier et al. 2003). Under heat stress conditions, they have shown to activate transcription and production of HSP genes and to modify maternal hormones and uterine environment (Ju 2005). Specifically, mRNA expression of *HSP70* has been reported to correlate with the length of heat shock to induce thermotolerance (Jia et al. 2010). Nevertheless, our results showed similar expression of *HSP70* and *HSP90*. Thus, we can suggest that as females were allocated to the climate chamber during three partums before the mRNA expression analysis; perhaps in this period, females might have adapted to our heat conditions (Omtvedt et al. 1971). In this way, the mRNA expression of erbB3 observed, involved in cell proliferation, differentiation and apoptosis during embryo development, was similar in both groups. Despite the important role of this gene, other studies have reported constant expression in different stages of embryo development and in endometrial tissue of different rabbit lines (Saenz-de-Juano et al. 2010; Llobat et al. 2011).

Therefore, our findings suggest a deleterious temperature effect on litter size and live born kits as a consequence of induced thermotolerance response on gene expression of pre-implantational embryo and endometrium associated with proliferation and differentiation and probably with implantation and uterine and foetal development during gestation.

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## Conflict of interest

None of the authors have any conflict of interest to declare.

## Author contributions

F Marco-Jiménez, C Natural-Alfonso, E Jiménez-Trigos and JS Vicente contributed to study design, analysed data and drafted paper. DS Peñaranda supported molecular technical assistance. FJ García-Diego contributed to design and control of the climatic chamber.

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