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Short communication: in-vitro effect of heat stress on bovine monocytes lifespan and polarization

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Short title: Effect of heat stress on bovine monocytes

Abstract

Heat stress (HS) has a negative impact on dairy cows' health, milk production, reproductive performance and immune defenses. Cellular and molecular responses to high temperatures in bovine polymorphonuclear cells and peripheral blood mononuclear cells (PBMCs) have been investigated so far. On the contrary, the effects of high temperatures on isolated monocytes remain almost undisclosed. The aim of this study was to unravel the *in vitro* effects of high temperatures, simulating a severe HS related body hyperthermia, on bovine lifespan and M1/M2 polarisation. The PBMCs were isolated from whole blood of 9 healthy dairy cattle. Monocytes were sorted by magnetic activated cell sorting and cultured over night at 39°C (normothermia) or 41°C (HS). Apoptotic rate and viability were assessed and mRNA abundance for heat shock proteins (*HSPs*), heat transcription factors (*HSFs*) and genes involved in monocyte/macrophage polarization (*STAT1, STAT2, STAT3, STAT6, IL1β, TGF1β, IL-10, COX2)* were quantified by qPCR. We found that apoptosis increased in monocytes exposed to 41°C, as compared to control, while viability conversely decreased. HS increased the abundance of *HSF1* and *HSP70.* The concomitant decrease of *STAT1* and *STAT2* and the increase of *STAT6* genes abundance at 41°C suggest, at transcriptional factors level, a polarization of monocytes from a classical activated M1 to a non-classically activated M2 monocytes. In conclusion, the exposure of bovine monocytes to high temperatures affects their lifespan as well as the abundance of genes involved in HS response and in monocyte/macrophages polarization phenotype, confirming that bovine immune response may be significantly affected by hyperthermia.

Keywords: Bovine; Heat shock; Immune response; Monocytes' polarization; qPCR

Introduction

The Intergovernmental Panel on Climate Change (IPCC, 2018) predicted that the rise in global average temperature may reach 1.5°C between 2030 and 2052, with an increase in number and intensity of heat waves and droughts. As a result, there are serious concerns about the risk for a decrease of animal performance, health and welfare, and about the overall sustainability of dairy production systems. High temperatures induce an imbalance between heat production and dissipation within the animal body (Das et al., 2016), increasing the risk of metabolic disorders (Ronchi et al., 2010) and infections (Olde Riekerink et al., 2007). Heat stress (HS) influences also the dairy cow welfare, as shown by panting and aggressiveness, and causes decline of milk yield and impairment of reproductive efficiency (Polsky and von Keyserlingk, 2017).

HS in livestock has also demonstrated an impact on immune responses. *In vivo*, *ex vivo* and *in vitro* studies pointed out that bovine peripheral blood mononuclear cells (PBMCs) proliferation is negatively affected by high temperatures (Jeong et al., 2014; Lacetera et al., 2009). Biological activities of bovine polimorphonuclear leukocytes (PMNs), including phagocytosis and reactive oxygen species (ROS) production (Lecchi et al., 2016), change as well after exposure to high temperatures.

Cellular response to stress, including heat related stress, induces the upregulation of molecular chaperones such as heat shock proteins (HSPs) and heat shock transcription factors (HSFs) (Gomez-Pastor et al., 2017; Lacetera et al., 2006). The HSPs complex play a role in livestock adaptation to HS, providing protection against hyperthermia and supporting the correct folding of stress denatured proteins. HSPs are classified into 5 families according to the molecular weight, including HSP27, HSP40, HSP60, HSP70, HSPS90 (Lianos et al., 2015). Heat stress induces the upregulation of HSP70 in bovine PBMCs (Bharati et al., 2017a) and of HSP90 and HSP27 in PBMC (Deb et al., 2014).

The HSF1 is an important mediator of the transcriptional response to HS involved in the thermotolerance and in the protection from apoptosis (Benjamin et al., 2002). Such action takes place by regulating HSP expression (Pirkkala et al., 2001), ROS homeostasis and cell cycle progression (Archana et al., 2017). In dairy cows, HSF1 is overexpressed in heat-stressed PBMCs (Gill et al., 2017).

In humans, hyperthermia induces a Th1/Th2 unbalance toward Th2 phenotype (Boneberg and Hartung, 2014). Results in heat stressed cows are consistent with what has been demonstrated in humans, as Th2-related-cytokine IL-10 is upregulated (Jeong et al., 2014) and Th1-associated-cytokine TNFα is downregulated (Amaral et al., 2009).

To the best of our knowledge, the effects of hyperthermia on bovine monocytes is unknown, in particular for what concerns the polarization toward M1/M2 lineage. Monocytes are bone marrow derived myeloid cells playing a pivotal role in defense against infection or injuries. Based on their inflammatory functions, at least two populations of monocytes can be defined, namely classical (M1) and non classical (M2) monocytes. M1 monocytes feature higher capabilities of phagocytosis and, more in general, a proinflammatory phenotype. M2 monocytes share a lower pro-inflammatory activity, although their precise physiological roles remain still undefined (Hussen and Schuberth, 2017).

This study was aimed at unraveling whether the exposure to high temperatures, mimicking conditions of HS related body hyperthermia, impacts on bovine monocyte apoptosis and viability and on the expression of genes related to the M1-M2 polarization.

Materials and method

Materials

Ethylenediaminetetraacetic acid disodium salt solution (EDTA) 0.5M, ficoll-Paque TM PLUS (GE Healthcare) sterile solution, red blood cell lysing buffer, sterile-filtered Dulbecco's Phosphate Buffered Saline without calcium and magnesium were purchased from SIGMA. RPMI 1640 Medium with Hepes and L-Glutamine, Non-essential Amino Acid Solution 100X, Penicillin Streptomycin Solution 100X and Fetal Bovine Serum were purchased from EuroClone. AlbuMAX TM II – Lipid Rich Bovine Serum Albumin was provided by Gibco – Life Technologies, sterile 24 wells plate from CytoOne, sterile 96 wells plate MICROTEST TM from Becton Dickinson, 384 well black plates from NUNC. CD14 MicroBeads, human 2ml, MS Columns and Pre-Separation filters 30μm were purchased from Miltenyi-Biotech. The complete cell medium was composed of RPMI 1640 medium, 10% FBS, 1% Non-essential Amino Acids and 1% antibiotics.

Sample collection and monocytes isolation

PBMCs were isolated from blood samples coming from diagnostic submissions to the Veterinary Hospital of Università degli Studi di Milano. Blood was collected from 9 pluriparous late lactating holstein friesian healthy cows from the same farm, at the end of the lactating period in sterile tubes containing 1.8mg K_2EDTA per ml of blood. CD14+ monocytes separation was performed using magnetic activated cell sorting (MACS), as previously reported (Ceciliani et al., 2007). Briefly, mononuclear cells were incubated with anti-human CD14 micro beads (Miltenyi-Biotech) for 15 min at 4 °C and CD14+ cells were isolated from a MD column (Miltenyi-Biotech) according to the manufacturer's instruction.The purity of the sorted cells (> 98%) was determined using an automatic cell counter (FACS Calibur cytometry system, Becton Dickinson, Mountain View, CA, U.S.A.) calibrated with Calibrite beads (Becton Dickinson). After monocytes isolation, cells were

incubated overnight at the physiological normothermic temperature of cows (39°C) and 41°C as heat stress condition already reported (Lacetera et al., 2009).

Viability assay (MTT)

The viability of CD14+ monocytes was assessed using Cell Proliferation Kit I (MTT) (Sigma-Aldrich). Briefly, 200 × 10^3 monocytes were seeded in triplicate in 96-well sterile plates at a final volume of 200 μl of medium. The plates were incubated overnight at 39°C and 41°C, respectively, in humidified atmosphere and 5% CO_2. Then, 20 μl of MTT labelling reagent were added in each well and incubated at 39°C for 4 h after incubation, following the manufacturer's instructions. The formazan crystals were solubilized by adding 100 μl of solubilizing buffer and incubating the plates overnight at 39°C. The absorbance was read at 550 nm with LabSystems Multiskan plate reader Spectrophotometer.

Apoptosis assay

The spontaneous apoptotic rate was measured in triplicate on 50 × 10^3 CD14+ monocytes in 384 wells black plates. Cells were incubated overnight at 39°C and 41°C, respectively, in humidified atmosphere and 5% CO_2. Apoptosis assay was carried out using the Apo-ONE® Homogeneous Caspase-3/7 kit (Promega) following the manufacturer's instruction. Twenty-five μl of the Caspase-3/7 reagent were added to each well and the fluorescence intensity was measured using a fluorescence plate reader Fluoroscan Ascent at 485/538 nm (absorbance/emission), every 30 minutes up to 4 hours.

Quantification of the mRNA abundance of genes involved in heat stress, inflammation and polarization toward M1/M2 lineage

CD14+ monocytes of each animal were seeded in triplicate in 24 wells sterile plates (1 x 10^6 cells/well) and incubated at 39°C and 41°C overnight. Total RNA was extracted from cells following the TRizol manufacturers' instructions. The quality and quantity of recovered RNA was assessed using a NanoDrop ND-1000 UV–vis spectrophotometer. Genomic DNA was eliminated using DNase I, RNase free kit (Fermentas, Life science) and reverse transcription was carried out on 1 μg RNA using iSCRIPT cDNA Synthesis kit (BIORAD). The cDNA was used as template to perform qPCR: each reaction, performed in duplicate in final volume of 15 μl, was composed of 7.5 μl of SsoFast™ EvaGreen Supermix (BIORAD) and primers (listed in Table 1). The thermal profile consisted of 95 °C for 10 min, 40 cycles of 95°C for 10s and 60, 61 or 61.5°C (see Table 1) for 30s; the melting curve was assessed by 80 cycles starting from 55°C with an increase of 0.5°C each 5s up to 95°C. The mRNA abundance of selected targets, namely *HSF1, HSP70 and HSP90AB1* belonging to heat shock proteins genes, *STAT1, STAT2* and *IL1β* involved in Th1 polarization, *STAT3, STAT6, TGF1β, IL-10* involved in Th2 polarization and *COX2* involved in inflammation processes was quantified. Three reference genes (*H3F3A*, *SF3A1* and *YWHAZ*) (Table 1) were identified and their stability was further evaluated by Maestro CFX Software (BioRad). The geometric mean of reference genes abundance was used for normalization. The relative quantification of genes of interest was carried out after normalization of the sample using the geometric mean of reference genes. The qPCR efficiency was evaluated by means of a relative standard curve (Table 1). A negative template control was included.The MIQE guidelines were followed as reported (Bustin et al., 2009).

Statistical analysis

Statistical analysis was carried out using software R version 3.5.3. Data normality distribution was determined by means of Shapiro Wilk test. Parametric paired t-test and

non-parametric paired Wilcoxon signed rank test were used to analyse normally and not normally distributed samples, respectively. Pearson or Spearman correlation tests were used to examine correlation between targets expression. Statistical significance was accepted at $P \leq 0.05$.

Results

Heat stress decreases viability and increases apoptosis of bovine CD14+ monocytes

Cells viability was assessed by MTT-assay. Results demonstrated that the viability of monocytes decreased at 41°C (P=0.04) (Figure 1).

Spontaneous apoptotic rate of monocytes is reported in Figure 2. Data are relative to the fluorescence produced by the specific cleavage of DEVD by two effectors caspases, namely caspase-3 and caspase-7, after overnight incubation at 41°C, as compared to 39°C control. Results showed that HS increased (P<0.001) the apoptotic rate of monocytes incubated at 41°C.

Heat stress modulates the mRNA abundance of genes involved in heat shock, inflammation and polarization toward M1/M2 lineage

In the second part of the study the issue whether high temperature regulates genes related to HS and polarization toward M1/M2 lineage was addressed. The results are presented in Figure 3. In detail, *HSF1* and *HSP70* mRNA were upregulated in monocytes incubated at 41°C as compared to the control ($HSF1_{41/39}$= 1.24, P=0.01; $HSP70_{41/39}$= 2; P=0.042), whereas no differences were found in the expression level of *HSP90B1*. The mRNA abundance of genes involved in inflammation (*COX2*) and in M1/M2 polarization (*STAT1, STAT2, STAT3, STAT6, IL1β, TGF1β* and *IL-10*), was also assessed. Heat stress did not affect the abundance of *COX2*, although a decreasing trend was appreciable at 41°C. The transcription factor *STAT6* was up-regulated ($ratio_{41/39}$ = 1.25; P=0.028), whereas *STAT1* and *STAT2* were down-regulated ($ratio_{39/41}$ = 2.26, P =0.007; $ratio_{39/41}$ = 1.36, P=0.013). Heat stress affected the abundance of *IL-10*, which decreased at 41°C (P=0.002), while didn't modulate *IL1-β* and *TGF1β* levels (P>0.05). A negative correlation between the expression of *HSP70* and *STAT1* (r = -0.71, P≤ 0.03) and

between *HSF1* and *STAT1* (r = -0.75, *P*≤ 0.02) and a positive correlation between *HSF1* and *STAT6* (r = 0.66, *P*≤ 0.04) was also found.

Discussion

Results reported herein present for the first time the effects that HS exerts on a sorted populations of monocytes in cattle. We tested apoptosis and viability, and the capability of high temperatures to modulate the gene abundance of HS related proteins, as well as transcription factors and cytokines related to polarisation toward M1/M2 lineage. Monocyte apoptosis increased and their viability decreased in heat stressed cells. Results are consistent with those from previous studies on not-sorted population of PBMCs, which demonstrated an increase of proapoptotic *Casp-3, Bcl-2, Bak, P53* mRNA abundance and the ratio of *Bax/Bcl-2* during summer season, and suggested a susceptibility of these cells to apoptosis (Somal et al., 2015). The present findings support the hypothesis that one of the several reasons linking HS to the development of diseases might be represented by decreasing viability of cells involved in immune defences, such as PBMCs (Lacetera et al., 2005) or neutrophils (Lecchi et al., 2016).

HSP related-genes, including *HSF1*, *HSP70* and *HSP90AB1*, have been previously demonstrated to be associated to HS adaptive response in bovine PBMCs (Bharati et al., 2017b). To the best of our knowledge, this is the first report describing the modulation of HSPs genes in bovine monocytes. The increase of HSPs related gene expression is consistent to what has been previously reported on unsorted PBMCs, as already reported in literature with the increase of HSP72 in heat stressed cells (Lacetera et al., 2006). HSP70 family is one of the central HSPs involved in HS, and an increase of their expression in both *in vitro* and *in vivo* studies after exposure to high temperatures has been reported (Bharati et al., 2017a). The activation of HSP related genes is also

confirmed by the upregulation of HSF1, which is one of the first functional participants involved in the coordination of the cellular response towards heat (Åkerfelt et al., 2007). HSF1 regulates many genes, including *HSPs* (Archana et al., 2017). During hyperthermia, the expression of HSPs is upregulated by the transcriptionally active form of HSF1 (Gill et al., 2017).

Diversity and plasticity are hallmarks of monocyte/macrophages that, in response to several signals from the microenvironment or under different pathophysiologic conditions, may acquire different phenotypes and polarize into M1 or M2 lineage. The M1 and M2 responses are related to opposing activities of phagocytosis and killing or repairing. M1 are stimulated by microbial molecules and produce pro-inflammatory cytokines, such as IL1β, IL-6, IL-12 and TNF-α, among others. Alternatively, M2 are stimulated by healing-type cues without infections and produce mainly anti-inflammatory cytokines, including TGFβ and IL-10, and protect against tissue damage (Chávez-galán et al., 2015). The regulation of polarization pathways toward M1/M2 lineage is of crucial importance in the development and resolution of inflammation, and involves several molecules including cytokines, signal transducers and activators of transcription. STAT signalling is a central pathway in modulating M1/M2 polarisation: the activation of transcription factors STAT1 and STAT2 drives monocye/macrophage function to the M1 phenotype, while STAT3 and STAT6 toward M2 phenotype (Chávez-galán et al., 2015). The present findings provide the evidence that HS induces a downregulation of *STAT1* and *STAT2,* jointly with a parallel upregulation of *STAT6,* suggesting a polarisation directed toward the M2-like phenotype at transcriptional level. Furthermore, *STAT1* and *STAT2* regulate the expression of several inflammatory genes including *COX2* and *IL1β,* while *STAT3* and *STAT6* regulate anti-inflammatory genes, such as *TGFβ* and *IL10* (Wang et al., 2014). The present result is in contrast with what has been previously reported on neutrophils of heat stressed cows (Jeong et al., 2014), indicating that HS was associated with an

upregulation of IL10. Also, this result is apparently in contrast with the finding that STAT6 and HSF1, both of them being involved in IL10 overexpression (Zhang et al., 2012), are equally upregulated. However, the role of IL10 in hyperthermia is poorly understood as reports from various studies among different animals species have been quite contradictory and inconsistent, showing instead a reduction in IL10 expression pattern in heat stressed rats. Moreover, all of these previous studies were carried out also on different cellular models, such as PMN (Jeong et al., 2014) and RAW264.7 (Zhang et al., 2012). In our study, the finding that *IL10* is not overexpressed is indirectly supported by the evidence that *STAT3*, which is activated by the ligand IL10-IL10R (Wang et al., 2014), is not modified during HS. Although it is difficult to suggest a clear reason to this apparent inconsistency with the literature, it can be speculated that monocytes needed a longer incubation time to induce an upregulation of *IL10* and to activate the pathways to switch to M2 lineage. *COX2* plays an essential role in inflammation (Moraes et al., 2015) and it is over-expressed by pro-inflammatory M1 macrophages. Moreover, we should not rule out potential differences related to cells, and species. Although not statistically significant, increasing incubation temperature induced a tendency to a decrease of *COX2*, supporting the hypothesis of a HS-induced polarization toward M2-like phenotype. M1 and M2 polarization represent extremes of a continuum in a wide range of activation states; macrophages polarization is highly dynamic, thus the *IL10* and *COX2* levels and *STAT3* steady-state could be explained as mixed intermediary phenotypes during HS.

The data presented in this study indicated that also in the bovine species high temperatures can affect monocytes lifespan and immune response. Conceivably, in a context of actions finalized to limit inflammatory events under conditions of HS, a priority for a down-regulation of proinflammatory cytokines is likely to be dictated by their dangerous biological activities (shock and tissue injury) at high concentrations.This may

be particularly important under conditions of HS, which place an increased demand on the cardiovascular system and is conducive to hypotension.

In the final part of the study, we reported a negative correlation between the abundance of *HSP70* and *STAT1* and *HSF1* and *STAT1,* and a positive correlation between *HSF1* and *STAT6*. The results of the correlation analysis are in line with previous findings, which identified HSPs as agents of an anti-inflammatory control actions at transcriptional factors level (Moseley, 1998).

Conclusion

Heat stress is negatively related to dairy cattle production and health, and decreases animal immune performances. In the present study, the impact of high temperature on the immune status was demonstrated through the decrease in viability and the increase of the apoptotic rate in CD14+ monocytes. Results reported herein also confirm the role of HSF1 and HSP70 on the HS adaptative response, and report for the first time the unbalance of bovine monocytes polarization toward M2-like phenotype at transcriptional factors level. Further studies should be carried out to elucidate the M1/M2 polarization after a prolonged HS exposure and to assess if the effect of high temperatures also induces lymphocyte switching, favouring an humoral immune response, or the polarization towards other immune cell subsets.

Ethics statement

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The procedures were carried out during routine disease testing

and out of the scope of Directive 2010/63/EU (art. 1.5.f “practices not likely to cause pain, suffering, distress or lasting harm equivalent to, or higher than, that caused by the introduction of a needle in accordance with good veterinary practice”). The protocol for care, handling, and sampling of animals defined in the present study was reviewed and approved by the Università degli Studi di Milano Animal Care and Use Committee (protocol no. 2/16).

Conflict of interest

None of the authors of this paper have a finantial or personal relationship with other people or organizations that would inappropriately influence or bias the content of the paper.

References

Åkerfelt, M., Trouillet, D., Erie, V.A.L., 2007. Heat Shock Factors at a Crossroad 27, 15–27. https://doi.org/10.1196/annals.1391.005

Amaral, B.C. do, Hayen, J., Bubolz, J., Tao, S., Dahl, G.E., Connor, E.E., 2009. Heat stress abatement during the dry period influences prolactin signaling in lymphocytes. Domest. Anim. Endocrinol. 38, 38–45. https://doi.org/10.1016/j.domaniend.2009.07.005

Archana, P., Aleena, J., Pragna, P., Vidya, M., 2017. Role of Heat Shock Proteins in Livestock Adaptation to Heat Stress. J. Dairy, Vet. Anim. Res. 5, 13–19. https://doi.org/10.15406/jdvar.2017.05.00127

Baumgard, L.H., Keating, A., Ross, J.W., Rhoads, R.P., 2015. Effects of heat stress on the immune system , metabolism and nutrient partitioning : implications on reproductive success. Rev. Bras. Reprod. Anim. 39, 173–183.

Benjamin, I.J., Xiao, X., Graves, K., McMillan, D.R., Shao, L., 2002. Targeted Disruption of Heat Shock Transcription Factor 1 Abolishes Thermotolerance and Protection against Heat-inducible Apoptosis. J. Biol. Chem. 273, 7523–7528. https://doi.org/10.1074/jbc.273.13.7523

Bharati, J., Dangi, S.S., Chouhan, V.S., Mishra, S.R., Bharti, M.K., Verma, V., 2017a. Expression dynamics of HSP70 during chronic heat stress in Tharparkar cattle. https://doi.org/10.1007/s00484-016-1281-1

Bharati, J., Dangi, S.S., Chouhan, V.S., Mishra, S.R., Bharti, M.K., Verma, V., Shankar, O., Yadav, V.P., Das, K., Paul, A., Bag, S., Maurya, V.P., Singh, G., Kumar, P., Sarkar, M., 2017b. Expression dynamics of HSP70 during chronic heat stress in Tharparkar cattle. Int. J. Biometeorol. 61, 1017–1027. https://doi.org/10.1007/s00484-016-1281-1

336 Boneberg, E.-M., Hartung, T., 2014. Febrile Temperatures Attenuate IL-1 Release by
337 Inhibiting Proteolytic Processing of the Proform and Influence Th1/Th2 Balance by
338 Favoring Th2 Cytokines. J. Immunol. 171, 664–668.
339 https://doi.org/10.4049/jimmunol.171.2.664
340 Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R.,
341 Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The
342 MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR
343 Experiments. Clin. Chem. 55, 611–622. https://doi.org/10.1373/clinchem.2008.112797
344 Ceciliani, F., Pocacqua, V., Miranda-Ribera, A., Bronzo, V., Lecchi, C., Sartorelli, P., 2007.
345 α1-Acid glycoprotein modulates apoptosis in bovine monocytes. Vet. Immunol.
346 Immunopathol. 116, 145–152. https://doi.org/10.1016/j.vetimm.2007.01.006
347 Chávez-galán, L., Olleros, M.L., Vesin, D., Garcia, I., Harris, R.A., 2015. Much more than
348 M1 and M2 macrophages , there are also CD169 + and TCR + macrophages 6, 1–15.
349 https://doi.org/10.3389/fimmu.2015.00263
350 Collier, R.J., Collier, J.L., Rhoads, R.P., Baumgard, L.H., 2008. Invited Review: Genes
351 Involved in the Bovine Heat Stress Response. J. Dairy Sci. 91, 445–454.
352 https://doi.org/10.3168/jds.2007-0540
353 Das, R., Sailo, L., Verma, N., Bharti, P., Saikia, J., Imtiwati, Kumar, R., 2016. Impact of
354 heat stress on health and performance of dairy animals: A review. Vet. World 9, 260–
355 268. https://doi.org/10.14202/vetworld.2016.260-268
356 Deb, R., Sajjanar, B., Singh, U., Kumar, S., Singh, R., Sengar, G., Sharma, A., 2014.
357 Effect of heat stress on the expression profile of Hsp90 among Sahiwal (Bos indicus)
358 and Frieswal (Bos indicus×Bos taurus) breed of cattle: A comparative study. Gene
359 536, 435–440. https://doi.org/10.1016/j.gene.2013.11.086
360 Gill, J.K., Arora, J.S., Sunil Kumar, B. V., Mukhopadhyay, C.S., Kaur, S., Kashyap, N.,
361 2017. Cellular thermotolerance is independent of HSF 1 expression in zebu and

crossbred non-lactating cattle. Int. J. Biometeorol. 61, 1687–1693. https://doi.org/10.1007/s00484-017-1350-0

Gomez-Pastor, R., Burchfiel, E.T., Thiele, D.J., 2017. Regulation of heat shock transcription factors and their roles in physiology and disease. Nat. Rev. Mol. Cell Biol. 19, 4.

Hussen, J., Schuberth, H.-J., 2017. Heterogeneity of Bovine Peripheral Blood Monocytes. Front. Immunol. 8, 1875. https://doi.org/10.3389/fimmu.2017.01875

Jeong, K.C., Tao, S., Thompson, I.M.T., Dahl, G.E., Monteiro, A.P.A., 2014. Effect of cooling during the dry period on immune response after Streptococcus uberis intramammary infection challenge of dairy cows. J. Dairy Sci. 97, 7426–7436. https://doi.org/10.3168/jds.2013-7621

Lacetera, N., Bernabucci, U., Basiricò, L., Morera, P., Nardone, A., 2009. Heat shock impairs DNA synthesis and down-regulates gene expression for leptin and Ob-Rb receptor in concanavalin A-stimulated bovine peripheral blood mononuclear cells. Vet. Immunol. Immunopathol. 127, 190–194. https://doi.org/10.1016/j.vetimm.2008.09.020

Lacetera, N., Bernabucci, U., Scalia, D., Basiricò, L., Morera, P., Nardone, A., 2006. Heat Stress Elicits Different Responses in Peripheral Blood Mononuclear Cells from Brown Swiss and Holstein Cows. J. Dairy Sci. 89, 4606–4612. https://doi.org/10.3168/jds.S0022-0302(06)72510-3

Lecchi, C., Dilda, F., Sartorelli, P., Ceciliani, F., 2012. Widespread expression of SAA and Hp RNA in bovine tissues after evaluation of suitable reference genes. Vet. Immunol. Immunopathol. 145, 556–562. https://doi.org/10.1016/j.vetimm.2011.12.017

Lecchi, C., Rota, N., Vitali, A., Ceciliani, F., Lacetera, N., 2016. In vitro assessment of the effects of temperature on phagocytosis, reactive oxygen species production and apoptosis in bovine polymorphonuclear cells. Vet. Immunol. Immunopathol. 182, 89–94. https://doi.org/10.1016/j.vetimm.2016.10.007

388 Lianos, G.D., Alexiou, G.A., Mangano, Alberto, Mangano, Alessandro, Rausei, S., Boni, L.,
389 Dionigi, G., Roukos, D.H., 2015. The role of heat shock proteins in cancer. Cancer
390 Lett. 1–5. https://doi.org/10.1016/j.canlet.2015.02.026
391 Moghaddam, A.S., Mohammadian, S., Vazini, H., Taghadosi, M., 2018. Macrophage
392 plasticity , polarization and function in health and disease † * Corresponding author :
393 Amirhossein Sahebkar , PharmD , PhD , Department of Medical Biotechnology ,
394 School of Medicine ,. https://doi.org/10.1002/jcp.26429
395 Moraes, C.N. De, Maia, L., Lima, P.F. De, Dias, M.C., Raposo-ferreira, T.M.M., Sudano,
396 M.J., Junior, J.B., Oba, E., 2015. Theriogenology and cyclooxygenase 2 messenger
397 RNA expression and prostaglandin F2 a receptor and cyclooxygenase 2 protein
398 expression in endometrial tissue from multiparous Nelore (Bos taurus indicus) cows
399 treated with cloprostenol sodium during puerperi. Theriogenology 83, 276–284.
400 https://doi.org/10.1016/j.theriogenology.2014.09.022
401 Moseley, P.L., 1998. Heat Shock Proteins and the Inflammatory Response. Ann. N. Y.
402 Acad. Sci. 856, 206–213. https://doi.org/10.1111/j.1749-6632.1998.tb08327.x
403 Olde Riekerink, R.G.M., Barkema, H.W., Stryhn, H., 2007. The Effect of Season on
404 Somatic Cell Count and the Incidence of Clinical Mastitis. J. Dairy Sci. 90, 1704–1715.
405 https://doi.org/10.3168/jds.2006-567
406 Pirkkala, L., Nykanen, P., Sistonen, L., 2001. Roles of the heat shock transcription factors
407 in regulation of the heat shock response and beyond. FASEB J. 15, 1118–1131.
408 Polsky, L., von Keyserlingk, M.A.G., 2017. Invited review: Effects of heat stress on dairy
409 cattle welfare. J. Dairy Sci. 100, 8645–8657. https://doi.org/10.3168/JDS.2017-12651
410 Puech, C., Dedieu, L., Chantal, I., Rodrigues, V., 2015. Design and evaluation of a unique
411 SYBR Green real-time RT-PCR assay for quantification of five major cytokines in
412 cattle, sheep and goats. BMC Vet. Res. 11, 65. https://doi.org/10.1186/s12917-015-
413 0382-0

Ronchi, B., Lacetera, N., Bernabucci, U., Nardone, A., Ranieri, M.S., 2010. Effects of climate changes on animal production and sustainability of livestock systems. Livest. Sci. 130, 57–69. https://doi.org/10.1016/j.livsci.2010.02.011

Somal, A., Aggarwal, A., Upadhyay, R.C., 2015. Effect of thermal stress on expression profile of apoptosis related genes in peripheral blood mononuclear cells of transition Sahiwal cow. Iran. J. Vet. Res. 16, 137–143.

Soriani, N., Panella, G., Calamari, L., 2013. Rumination time during the summer season and its relationships with metabolic conditions and milk production. J. Dairy Sci. 96, 5082–5094. https://doi.org/10.3168/jds.2013-6620

Wang, N., Liang, H., Zen, K., 2014. Molecular mechanisms that influence the macrophage M1 – M2 polarization balance 5, 1–10. https://doi.org/10.3389/fimmu.2014.00614

Zhang, H., Zhang, L., Yu, F., Liu, Y., Liang, Q., Deng, G., Chen, G., Liu, M., Xiao, X., 2012. HSF1 Is a Transcriptional Activator of IL-10 Gene Expression in RAW264 . 7 Macrophages 35, 1558–1566. https://doi.org/10.1007/s10753-012-9471-4

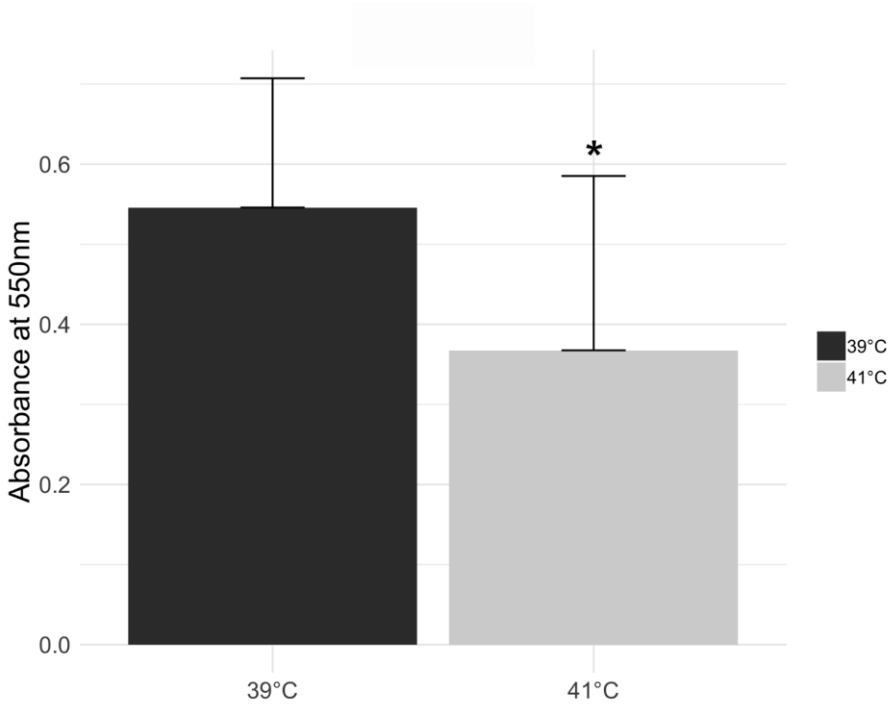
Table 1: *Sequences of oligonucleotide primers used in the current study.*
YWHAZ and SF3A1 primers sequences were from (Lecchi et al., 2012)*; H3F3A primer were from Puech et al., 2015* (Puech et al., 2015)*; the other primers were designed on the basis of GenBank sequences.*

Symbol and accession number		Sequence	Primer concentration (nM)	PCR Efficiency (%) and regression coefficient (r2)	Amplicon length (bp)	Annealing T°C
H3F3A NM_001014389.2	Forward 5'→3'	CGCAAACTTCCCTTCCAGCGTC	400	90.6 0.997	102	61
	Reverse 5'→3'	TCACTTGCCTCCTGCAAAGCAC				
YWHAZ XM_025001429.1	Forward 5'→3'	GCATCCCACAGACTATTTCC	400	98.4 0.993	119	61.5
	Reverse 5'→3'	GCAAAGACAATGACAGACCA				
SF3A1 NM 001081510	Forward 5'→3'	CCTTACCATGCCTACTACCGG	300	104.5 0.996	144	61.5
	Reverse 5'→3'	CACTTGGGCTTGAACCTTCTG				
STAT1 NM_001077900.1	Forward 5'→3'	AGCAAGCCTTATGGGACCGCAC	400	109.4 0.997	81	61.5
	Reverse 5'→3'	TGCAGGGCTGTCTTTCCACCAC				
STAT2 NM_001205689.1	Forward 5'→3'	TCATGCCAAACGGTGATCCAG	300	102.7 0.992	82	61.5
	Reverse 5'→3'	GCATAGAAGTGGCTGGGGTTG				
STAT3 NM_001012671.2	Forward 5'→3'	AACGTGGGATCAAGTGGCCGAG	300	108.2 0.996	97	61
	Reverse 5'→3'	TTTCTCCGCCAGCGTCGTCAAC				

STAT6 NM_001205501.1	Forward 5'→3'	ACCATTGCCCACGTCATCCGAG	300	94.4 0.995	127	61
	Reverse 5'→3'	TTTTGAGCTGAGCGAGGTCCCG				
IL-10 NM_174088.1	Forward 5'→3'	GGAGAAGCTGAAGACCCTGCG	300	109.8 0.991	77	61.5
	Reverse 5'→3'	CCGCCTTGCTCTTGTTTTCGC				
IL-1B NM_174093.1	Forward 5'→3'	TAGCGGAGAAGGCAATGGCACC	350	93.5 0.996	109	61.5
	Reverse 5'→3'	TCGTGTCCGACTCTTAGCGACC				
TGFB1 NM_001166068.1	Forward 5'→3'	TCACCCGCGTGCTAATGGTG	250	102.7 0.995	136	61.5
	Reverse 5'→3'	GCCCGAGAGAGCAACACAGG				
HSP70 NM_203322.3	Forward 5'→3'	TCCGTGAGAACAGCTTCCGCAG	350	98.2 0.993	89	60
	Reverse 5'→3'	AACGGCCCACAGGATCAACGAC				
HSP90AB1 NM_001079637.1	Forward 5'→3'	AACGACAAGGCCGTCAAGGACC	300	93.9 0.995	138	61
	Reverse 5'→3'	TTCATCAATGCCCAGGCCGAGC				
HSF1 NM_001076809.1	Forward 5'→3'	AGTTTGCCAAGGAGGTGCTGCC	300	99.3 0.996	74	61
	Reverse 5'→3'	CATGTTGAGCTGCCGCACGAAG				
COX2 NM_174445.2	Forward 5'→3'	TGGCATCCCCTTCTGCCTGACG	450	98.6 0.997	159	61.5
	Reverse 5'→3'	ATTCCTACCGCCAGCGACCCTG				

435

436 **Figures**



437

438 **Figure 1**: monocytes viability after 39°C and 41°C incubation. Samples are run in
439 triplicates. Data are means ± SEM of 9 independent experiments, using a total of 9
440 animals * indicates when $P \leq 0.05$

441

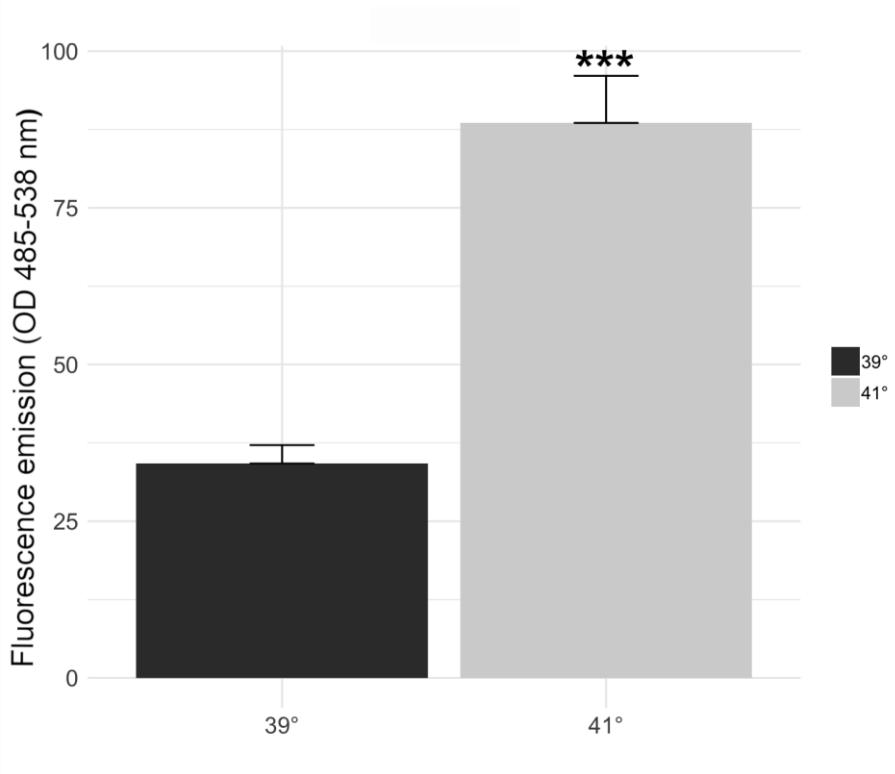


Figure 2: monocytes apoptosis after 39°C and 41°C incubation. Samples are run in triplicates. Data are means ± SEM of 9 independent experiments, using a total of 9 animals. *** indicates when *P*<0.001

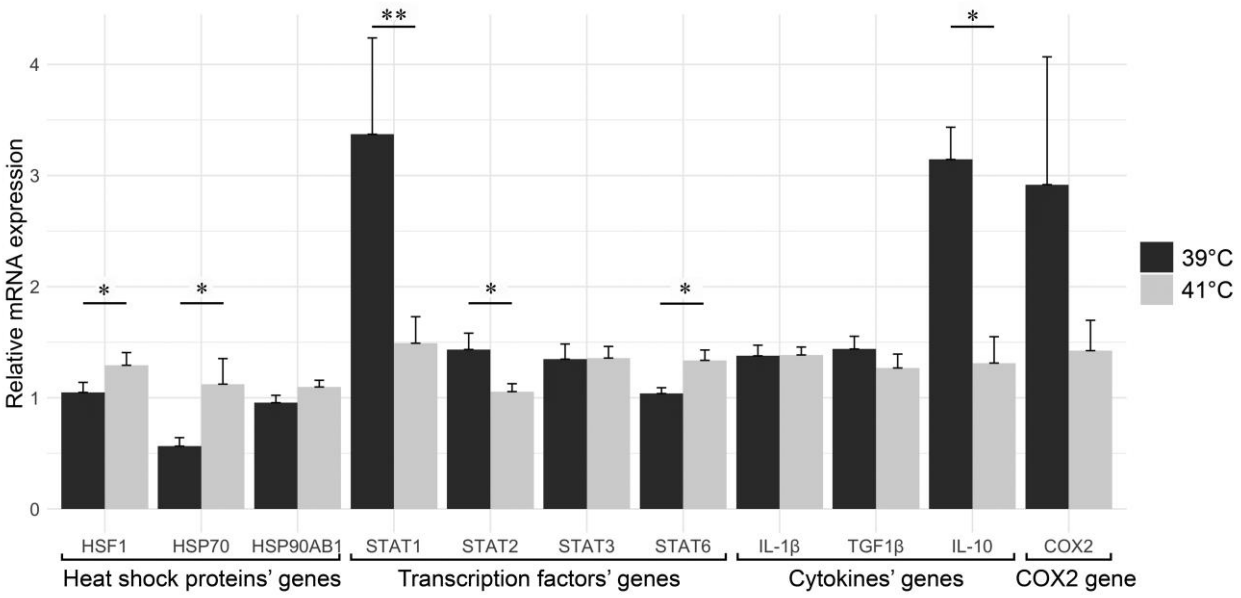


Figure 3: Relative mRNA expression of 11 targets in monocytes after 39°C and 41°C incubation. Data are means ± SEM of 9 independent experiments, using a total of 9 animals. * indicates when $P<0.05$; ** indicates when $P<0.01$