Heat stress induces oxidative stress and activates the KEAP1-NFE2L2-ARE pathway in reproduction-related cells

Research Article

Hanako Bai[1,*,†], Manabu Kawahara[1], Kazuya Kusama[2], Toshihiro Sakurai[3], Christiane Pfarrer[4], Masashi Takahashi[1]

[1)] *Laboratory of Animal Breeding and Reproduction, Research Faculty of Agriculture, Hokkaido University, Kita-ku Kita 9 Nishi 9, Sapporo 060-8589, Japan*

[2)] *Department of Endocrine Pharmacology, Tokyo University of Pharmacy and Life Sciences, Tokyo 192-0392, Japan*

[3)] *School of Pharmaceutical Science, Ohu University, 31-1 Misumido, Tomita-machi, Koriyama-shi, Fukushima 963-8611, Japan Tokyo University of Pharmacy and Life Sciences*

[4)] *Institute for Anatomy, University of Veterinary Medicine Hannover, Foundation Bischofsholer Damm 15 30173 Hannover, Germany*

[†] **Correspondence:**

H. Bai (e-mail: hbai@agr.hokudai.ac.jp)

ABSTRACT

Heat stress negatively affects the reproductive function of in animals and humans. Although a relationship between heat and oxidative stress has been suggested, the underlying mechanism has not been sufficiently examined in reproduction-related cells. Therefore, we aimed to investigate whether heat stress induces oxidative stress using a variety of reproduction-related cells including bovine placental and cumulus-granulosa cells, human cell lines derived from cervical and endometrial cancers, and fibroblasts derived from endometrium. Quantitative polymerase chain reaction analysis showed that the expression levels of representative heat and oxidative stress-related genes were significantly increased in cells cultured at high temperatures compared with those in cells cultured at basal temperatures. Moreover, luciferase reporter assays showed that the reporter activity of the heat shock element and antioxidant responsive element (ARE) was increased in cells cultured at high temperatures compared to that in cells cultured at basal temperatures. Furthermore, the stability of nuclear factor erythroid 2 like 2 (NFE2L2), a master regulator of the cellular stress response, increased under high temperatures. Point mutations in Kelch-like ECH-associated protein 1 (KEAP1) cysteine residues reduced the luciferase activity. Our results suggest that heat stress induces oxidative stress and that the KEAP1-NFE2L2-ARE pathway may play a protective role in reproduction-related cells against heat stress.

Keywords: *gene expression, heat stress, oxidative stress*

INTRODUCTION

Heat stress negatively affects several reproductive functions in animals and humans, including oocyte maturation, fertilization, and embryonic development (Kadokawa *et al*., 2012; Roth, 2015; Sakatani, 2017; Boni, 2019; Cowell et al., 2023). Heat stress causes considerable economic losses in the dairy industry (Boni, 2019); as such, these negative effects of heat stress on the physiological condition of cows is well studied. In winter, bovine average body temperature is 38.8 ± 0.04 °C, which increases to 39.3 ± 0.03 °C in summer (Sartori *et al*., 2002). In addition, uterine temperatures are approximately 0.2 °C higher than rectal temperatures (El-Sheikh *et al*., 2013). High temperatures reduce blood flow to the uterus (Gwazdauskas *et al*., 1975; Roman-Ponce *et al*., 1978), and maternal heat stress negatively affects the fetal viability in utero and offspring (Tao *et al*., 2012; Monteiro *et al*., 2016a, 2016b). Moreover, heat stress disrupts appropriate secretion of prostaglandin (PG) E2 and/or PGF2α in endometrial cells that are important for corpus luteum function, which supports pregnancy (Putney *et al*., 1988; Sakai *et al*., 2018). In this respect, the conception rate of artificial insemination decreases in the summer (García-Ispierto *et al*., 2006; Huang *et al*., 2009; De Rensis *et al*., 2017; Oikawa *et al*., 2019).

Heat shock proteins (HSPs) are transcriptionally activated under heat stress conditions and are thus used as heat stress markers (Binder, 2014). They are expressed in various reproductive cells and tissues, such as oocytes, the placenta, and the endometrium (Neuer *et al*., 2000), are crucial for the heat stress response, and protect against cellular damage. Among the HSPs, HSP70 and HSP90 play central roles in the heat stress response and regulation of protein misfolding in various cells and tissues. Transcriptional activation is mediated by heat shock factors (HSFs) that bind to conserved heat shock responsive DNA elements (HSE) (Wu, 1995; Morimoto, 1998; Binder, 2014). HSFs have been identified as heat stress-inducible regulators of HSP genes; however, they are induced by various stressors, including oxidative stress (Barna *et al*., 2018). Heat stress induces oxidative stress and the expression of its marker genes, such as superoxide dismutase (SOD) and catalase, in the plasma (Bernabucci *et al*., 2002). Additionally,

heat stress-induced oxidative stress agents, such as reactive oxygen species (ROS), impair cellular functions in bovine ovaries and embryos (Roth, 2015; Sakatani, 2017). The information provided in these studies indicates a close relationship between heat and oxidative stress.

The transcription factor nuclear factor erythroid 2 like 2 (NFE2L2, also known as NRF2) is a master regulator of the cellular oxidative stress response. NFE2L2 is associated with ROS homeostasis by regulating the antioxidant defense mechanisms (Ishii *et al*., 2000; Suzuki *et al*., 2013). The NFE2L2 target genes have a DNA sequence known as the antioxidant response element (ARE), which is involved in the homeostatic control of oxidants (Rushmore *et al*., 1991; Jin *et al*., 2016; Liang *et al*., 2019). Under basal conditions, Kelch-like ECH-associated protein 1 (KEAP1) binds to NFE2L2 and sequesters it from the nucleus, thereby preventing the activation of its target gene. Under stressed conditions, NFE2L2 released from KEAP1 escapes proteasomal degradation, stabilizes and translocates into the nucleus, and activates its target genes (Itoh *et al*., 1999; Ishii *et al*., 2000; Kobayashi *et al*., 2004; Suzuki *et al*., 2013). This KEAP1-NFE2L2 system is a conserved defense mechanism against oxidative stress. KEAP1 is a cysteine-rich protein, and the significance of its cysteine residues has been demonstrated using site-directed mutagenesis (Saito *et al*., 2016; Suzuki *et al*., 2019). Three cysteine residues (Cys151, Cys273, and Cys288) are important in environmental stress sensing (Saito *et al*., 2016), and KEAP1 uses four cysteine residues (Cys226, Cys613, Cys622, and Cys624) to sense H_2O_2 (Suzuki *et al*., 2019).

We previously showed that the KEAP1–NFE2L2 pathway was involved in oxidative stress resistance under heat-stressed culture conditions in bovine endometrial epithelial cells (Murata *et al*., 2021), and it also responds to oxidative stress during embryonic development (Amin *et al*., 2014). Moreover, the KEAP1-NFE2L2 pathway may be involved in oxidative stress resistance in the other reproduction-related cells; however, this has not yet been comprehensively investigated. Therefore, in this study, we aimed to examine the effects of heat stress on reproduction-related cells using several cell lines to better understand the cellular functions under heat stress. We examined the effects of heat stress on a variety of reproduction-related cells including bovine placental and cumulus-granulosa cells, human cell lines derived from cervical

and endometrial cancers, and human fibroblasts derived from the endometrium.

MATERIALS AND METHODS

Cell culture

Bovine placental F3 cells (Hambruch *et al*., 2010) and bovine cumulus granulosa (CG) cells (Ho *et al*., 2021) were cultured as described previously. Briefly, F3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan)/Ham's F12 medium supplemented with 10% (v/v) fetal bovine serum (FBS; BioWest, Funakoshi Co., Ltd., Tokyo, Japan), and antibiotic-antimycotic solution (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C with 5% CO_2. Bovine CG cells were cultured in DMEM supplemented with 10% FBS and antibiotic-antimycotic solution at 37 °C with 5% CO_2. Human cell line derived from cervical cancer (HeLa, RCB0007) (Gey *et al*., 1952), human cell line derived from endometrial cancer (HHUA, RCB0658) (Ishiwata *et al*., 1984), and human fibroblasts derived from endometrium (HOEF, RCB1010) were obtained from the Riken Cell Bank (Tsukuba, Japan) and cultured in DMEM with 10% FBS at 37 °C with 5% CO_2. Cells were cultured under standard conditions of 37 °C, 5% CO_2, and saturated humidity, and they were then cultured at varying temperatures: 37 °C (cell establishment temperature), 38.5 °C (normal bovine body temperature), and 40.5 °C (bovine body temperature under high environmental temperature) for 12 h. The same temperature setting was used for both bovine and human cells because the heat stress experiments were previously conducted under similar temperature conditions (normothermia, mild, and severe hyperthermia [37 °C, 39 °C, and 41 °C, respectively]) in an *in vitro* cell culture system (McCormick *et al*., 2021). In our previous studies, the 12 h duration for heat-stressed culture was chosen as the typical length of a day, and since this has been shown to induce HSPs gene expressions, we set 12 h for investigating the mechanism of the heat stress response.

RNA analysis

Total RNA was extracted from cells using NucleoSpin RNA Plus (Takara Bio UK Ltd.,

Shiga, Japan) following the manufacturer's protocol. Reverse transcription to cDNA and subsequent quantitative real-time PCR (qPCR) analyses were performed as previously described (Murata *et al*., 2021) using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan) and THUNDERBIRD™ SYBR® qPCR Mix (Toyobo) following the manufacturer's instructions. Target gene expression levels were determined by qPCR using a LightCycler 96 (Roche Diagnostics, Basel, Switzerland). The primer details are listed in Table 1. The thermal cycling conditions comprised 1 cycle at 95 °C for 30 s, followed by 50 cycles at 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 30 s. The relative mRNA abundance was calculated based on the expression levels of *H2AFZ*, which was used as a reference gene as it showed stable expression in bovine endometrium and was relatively stable under heat stress conditions (Bai *et al*., 2020; Murata *et al*., 2021). Each run included melting curve analysis to ensure amplification specificity and the absence of primer dimer formation. All qPCR experiments were performed in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin *et al*., 2009).

Reporter assay

Reporter constructions containing HSE and ARE sequences have been described previously (Murata *et al*., 2021). Reporter constructs containing HSE and ARE were generated using the primers listed in Table 2. The HSE or ARE fragments were inserted into the pNL [NLucP/minP/Hygro] vector (Promega, Madison, WI, USA). The cells were seeded in a 4-well plate (Thermo Fisher Scientific). At 70–80% confluency, 100 ng pNL (HSE, ARE) reporter constructs, 5 ng pGL4.53 [luc2/PGK] firefly reporter (used to correct for transfection efficiency), and 2.0 μL ViaFect™ Transfection Reagent (Promega) were prepared in 10 μL DMEM without supplements.

The pNLF1-NRF2 [CMV/neo] reporter vector (Promega) was used to evaluate NFE2L2 (NRF2) stability. The product includes NRF2 vector (GenBank accession number: KF853602) and KEAP1 vector (GenBank accession number: KF853604) harboring sequences based on

human sequences. Site-directed mutagenesis of the pKEAP1 vector was performed by Kazusa Genome Technologies, Inc. (Chiba, Japan). The pGL4.53 [luc2/PGK] firefly reporter (5 ng), pNLF1-NRF2 vector (10 ng) along with the pKEAP1 vector (100 ng) and 2.0 μL ViaFect™ Transfection Reagent was prepared in 10 μL DMEM without supplements. The amount of transfected DNA in the KEAP1-free group was corrected using a Transfection Carrier DNA (Promega).

After 15 min of incubation, the plasmid mixture was added to the cells and incubated at 37.0 °C under 5% CO_2 for 3 h. After 3 h of incubation, the medium was replaced with a serum-containing medium. After 12 h of transfection, cells were further incubated at 37.0 °C, 38.5 °C, and 40.5 °C for 12 h under 5% CO_2. These cells were then lysed by the addition of 50 μL Passive Lysis Buffer (Promega). The luciferase assay was performed using the Nano-Glo® Dual-Luciferase® Reporter Assay System (Promega). Each sample was analyzed in duplicate per group (n = 9). For site-directed mutagenesis experiments, HeLa and HHUA cells were selected due to transfection stability.

Statistical analyses

Statistical analyses were conducted using the R software package (version 3.6.2) (https://www.r-project.org/), and data were analyzed using a Student's *t*-test to compare the control and heat stress groups. A one-way analysis of variance, followed by the Tukey–Kramer test, was used to make multiple comparisons among groups. Statistical significance was set at $P < 0.05$, and the results are presented as mean ± standard error of the mean (SEM) obtained from six or more independent experiments.

RESULTS

Effect of heat-stressed culture on *heat shock protein* (*HSP*) *70* and *HSP90* expression in reproduction-related cells

qPCR analyses were conducted to examine the expression levels of heat stress marker genes *HSP70* and *HSP90* in cells cultured at varying temperatures: 37 °C (cell establishment temperature), 38.5 °C (normal bovine body temperature), and 40.5 °C (bovine body temperature under high environmental temperature) (Figure. 1). In human cells, these temperatures correspond to normal, mild, and severe hyperthermia, respectively. The expression levels of *HSP70* and *HSP90* gradually increased under increased temperature conditions, significantly increasing at 40.5 °C compared with the 37 °C groups ($P < 0.05$). No significant differences in HSP expression were observed between the 37 °C and 38.5 °C groups.

Effect of heat stress on HSE and ARE transcriptional activity and expression levels of antioxidant enzyme genes

The effect of heat stress on HSE and ARE activities in cells cultured at varying temperatures was examined using a luciferase reporter assay (Figure 2). HSE and ARE are response elements for heat and oxidative stress, respectively. In cells cultured under heat stress conditions of 40.5 °C for 12 h, both HSE and ARE activities were elevated compared with control cells cultured at 37 °C ($P < 0.05$), except when ARE was transfected into bovine CG and human HOEF cells. Additionally, NFE2L2 stability was significantly increased in cells cultured under heat stress conditions of 40.5 °C ($P < 0.05$).

The expression levels of the antioxidant enzyme genes, *Catalase*, copper/zinc SOD (*CuZnSOD*), and manganese SOD (*MnSOD*), which are possible targets of NFE2L2, were examined using qPCR (Figure 3). The expression levels of *Catalase* were increased in cells cultured at 40.5 °C under heat stress conditions compared to control cells cultured at 37 °C ($P < 0.05$), except in HeLa cells. *CuZnSOD* expression increased in cells cultured at 40.5 °C under heat stress conditions compared with control cells cultured at 37 °C ($P < 0.05$), except in HHUA cells. The *MnSOD* expression levels also rose with increasing temperature in all cells, with significant differences observed in bovine CG and F3 cells cultured at 40.5 °C.

Moreover, the expression levels of *KEAP1* and *NFE2L2* mRNA slightly increased under

heat stress conditions; however, significant differences were observed only for *KEAP*1 in CG and F3 cells and *NFE2L2* in HOEF cells ($P < 0.05$) (Supplementary Figure 1).

Effect of cysteine residues site mutation on the degree of NFE2L2-reporter activity

Multiple cysteine residues exist in the KEAP1 protein. Among these, we focused on three cysteine residues (Cys151, Cys273, and Cys288) critical in stress sensing (Saito *et al*., 2016) and on four cysteine residues (Cys226, Cys613, Cys622, and Cys624) that sense H_2O_2 (Suzuki *et al*., 2019); we prepared the cells harboring point-mutations corresponding to one of the two types of residue groups ($KEAP1^{C151/273/288mt}$ and $KEAP1^{C226/613/622/624mt}$) for the following experiments (Figure 4). Both the KEAP1 mutations at three cysteine residues (Cys151, Cys273, and Cys288) ($KEAP1^{C151/273/288mt}$) and four cysteine residues (Cys226, Cys613, Cys622, and Cys624) ($KEAP1^{C226/613/622/624mt}$) suppressed NFE2L2 reporter activity, as did intact KEAP1 ($KEAP1^{WT}$) under basal culture conditions ($P < 0.05$). NFE2L2 stability was significantly increased in cells cultured under heat stress conditions of 40.5 °C compared with control cells cultured at 37 °C ($P < 0.01$). Under heat stress conditions, the mutant $KEAP1^{C151/273/288mt}$ suppressed NFE2L2 reporter activity as well as $KEAP1^{WT}$ ($P < 0.05$). Under heat stress, the mutant $KEAP1^{C226/613/622/624mt}$ suppressed NFE2L2 reporter activity to a greater extent than did $KEAP1^{WT}$.

DISCUSSION

We examined the expression levels of HSPs mRNA in cells cultured at varying temperatures: 37 °C, 38.5 °C, and 40.5 °C. HSPs are heat stress markers that play a protective role against high temperatures in various reproductive cells and tissues (Neuer *et al*., 2000). Notably, HSP expression levels were significantly higher in cells cultured at 40.5 °C compared with those at 37 °C and 38.5 °C. Typically, the established temperature for most mammalian cells, including bovine cells is 37 °C, close to human body temperature. On the other hand, bovine body temperature is approximately 38.5 °C, and it increases under high-temperature conditions (Sartori

et al., 2002); therefore, numerous studies on heat stress have used either 37 °C or 38.5 °C as control temperatures to compare the effects with those observed at 40 °C or higher (Rivera and Hansen, 2001; Li *et al*., 2015; Rąpała *et al*., 2018; Sakai *et al*., 2018; Singh *et al*., 2020). Notably, no significant difference was observed in HSP expression at 37 °C and 38.5 °C; however, a significant increase was observed at 40 °C. Additionally, evident differences in the gene expressions of antioxidant enzymes at 40.5 °C were observed using qPCR, and evident differences in the activities of HSE, ARE, and NFE2L2 were observed at 40.5 °C using reporter assays. These results indicate that 40.5°C for 12 h of heat stress can induce a heat stress response in both cow and human cells. It is unclear whether 37 °C or 38.5 °C is the better control temperature for bovine cells; however, more detailed analysis involving more detailed gene expression is needed. For the luciferase assay with the KEAP1 mutant reporter in this study, 37 °C and 40.5 °C were selected for comparison to obtain definitive results.

In this study, the reporter activities of HSE, ARE, and NFE2L2 were significantly higher in the reproduction-related cells cultured under heat stress conditions of 40.5 °C than under control conditions. Moreover, the mRNA expression levels of antioxidant enzymes that are possible targets of NFE2L2 gradually increased and were significantly high at 40.5 °C heat stress than those at 37°C and 38.5°C. These results are consistent with our previous results from heat stress experiments using bovine endometrial epithelial cells (Murata *et al*., 2021), and they suggest that antioxidant enzyme genes could be upregulated in response to oxidative stress via stabilization of NFE2L2 in a variety of mammalian reproduction-related cells. Although there have been several excellent studies in the past using mutants of KEAP1 (Zhang *et al*., 2003; Kobayashi *et al*., 2004; Saito *et al*., 2016), this is the first study to examine its effects on reproduction-related cells under heat-stressed conditions.

Among the multiple cysteine residues in KEAP1, we focused on three (Cys151, Cys273, and Cys288) (Saito *et al*., 2016) and four (Cys226, Cys613, Cys622, and Cys624) (Suzuki *et al*., 2019). These KEAP1 mutants, KEAP1$^{C151/273/288mt}$ and KEAP1$^{C226/613/622/624mt}$, retained equivalent efficacy in repressing NFE2L2 reporter activity to KEAP1^{WT} under basal culture conditions.

In human HHUA cells under heat stress conditions, both KEAP1$^{C151/273/288mt}$ and KEAP1$^{C226/613/622/624mt}$ suppressed NFE2L2 reporter activity as well as KEAP1^{WT}, but the suppressive effect on NFE2L2 reporter activity of KEAP1$^{C226/613/622/624mt}$ was relatively greater than that of KEAP1$^{C151/273/288mt}$. In human HeLa cells under heat culture conditions, transfection of KEAP1^{WT} and KEAP1$^{C151/273/288mt}$ increased NFE2L2 stability, as did the non-KEAP1 transfected cells. The suppressive effect was observed only in the KEAP1$^{C226/613/622/624mt}$ transfection. These results suggest that the cysteine residues C226/613/622/624 of KEAP1 may play a role in sensing heat and oxidative stress. The underlying mechanism for the different responses in the two cell types is unclear, but it is possible that other factors, besides KEAP1, contribute to NFE2L2 stabilization in response to heat-oxidative stress, or that variations in endogenous KEAP1 expression play a role.

Many studies have reported the effects of oxidative stress on reproductive function in animals and humans. For example, ROS affects multiple physiological processes, including oocyte maturation, fertilization, embryonic development, and pregnancy (Agarwal *et al*., 2005). Oxidative stress is involved in preeclampsia, hydatidiform moles, birth defects, and abortion. In the present study, the induction of oxidative stress by heat stress was observed in reproduction-related cells, the reporter activities of HSE, ARE, and NFE2L2 were increased by heat stress, while heat stress also induced the expression of ARE-dependent antioxidant enzyme genes. These results suggest that the KEAP1-NFE2L2-ARE pathway plays a protective role in cells cultured under heat stress. Modulation of the KEAP1-NFE2L2-ARE pathway may therefore help to reduce the adverse effects of heat and oxidative stress on mammalian reproductive cells and their functions.

This research has certain limitations. First, the experiments were conducted using an *in vitro* culture system, which may not fully represent *in vivo* tissue and cellular conditions. Second, as previously noted, endogenous KEAP1 expression could have influenced the outcomes. In addition, only a limited number of cells were used in this study, and in particular, only two types of human cells were used in the reporter assay with mutant KEAP1 due to the stability of

transfection efficiency. Therefore, these findings should be interpreted with caution, and additional experiments involving diverse cell types are required to compare and validate responses across different tissue types, cell types, or species.

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Conflict of interest

The authors declare no conflicts of interest for this article.

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Figure legends

Figure 1. Effect of heat stress on HSP gene expression in cultured cells.
Relative gene expressions of heat shock proteins *HSP70* and *HSP90* under varying culture temperatures (37 °C, 38.5 °C, and 40.5 °C, 12 h) were examined using qPCR. Bovine ovary cumulus granulosa cells (CG) (A), fetal placenta-derived F3 cells (B), human cell line derived from cervical cancer (HeLa) cells (C), human endometrial carcinoma cell line (HHUA) (D), and human fibroblasts derived from endometrial (HOEF) cells (E) were used (n = 6, each cell). The expression of *H2AFZ* was used as an internal control. Data are presented as means ± standard error of the mean (SEM). Different letters (a, b, c) indicate significant differences between the groups ($P < 0.05$).

Figure 2. Heat stress induces transactivation of the heat shock responsive element (HRE) and the antioxidant response element (ARE) in *in vitro* cultured cells.
Activation of the reporter construct transfected with HSE- (Light) or ARE- (Middle) reporter

construct into reproduction-related cells and cultured under varying culture temperatures (37 °C, 38.5 °C, and 40.5 °C, 12 h). The stability of the NFE2L2 reporter construct co-transfected with the KEAP1 construct into cells and cultured at varying temperatures (left).

Bovine CG (A), F3 (B), human HeLa (C), HHUA (D), and HOEF (E) were used (n = 8, each cell). Data are presented as means ±SEM. Different letters (a, b, c) indicate significant differences between the groups ($P < 0.05$).

Figure 3. Effect of heat stress on antioxidant enzyme gene expression in cultured cells.

Relative expressions of antioxidant enzyme genes catalase, copper/zinc superoxide dismutase (*CuZnSOD*), and manganese superoxide dismutase (*MnSOD*), which are possible targets of NFE2L2, were examined using qPCR at varying culture temperatures (37 °C, 38.5 °C, and 40.5 °C, 12 h). The expression levels of *H2AFZ* were used as an internal control. Bovine CG (A), F3 (B), human HeLa (C), HHUA (D), and HOEF (E) were used (n = 6, each cell). Data are presented as means ± standard error of the mean (SEM). Different letters (a, b, c) indicate significant differences between the groups ($P < 0.05$).

Figure 4. Effect of intact or mutation KEAP1 on NFE2L2 stability.

A. Cysteine residues in KEAP1 examined in this study. HeLa (B) and HHUA cells (C) were transfected with NFE2L2-reporter with KEAP1 intact ($KEAP1^{WT}$) or cysteine-mutated construct (n = 9, each cell). Data are presented as means ± standard error of the mean (SEM). Different letters (a, b, c: Control group; A, B, C: heat stress group) indicate significant differences between groups ($P < 0.05$). ** $P < 0.01$.

Figure 1

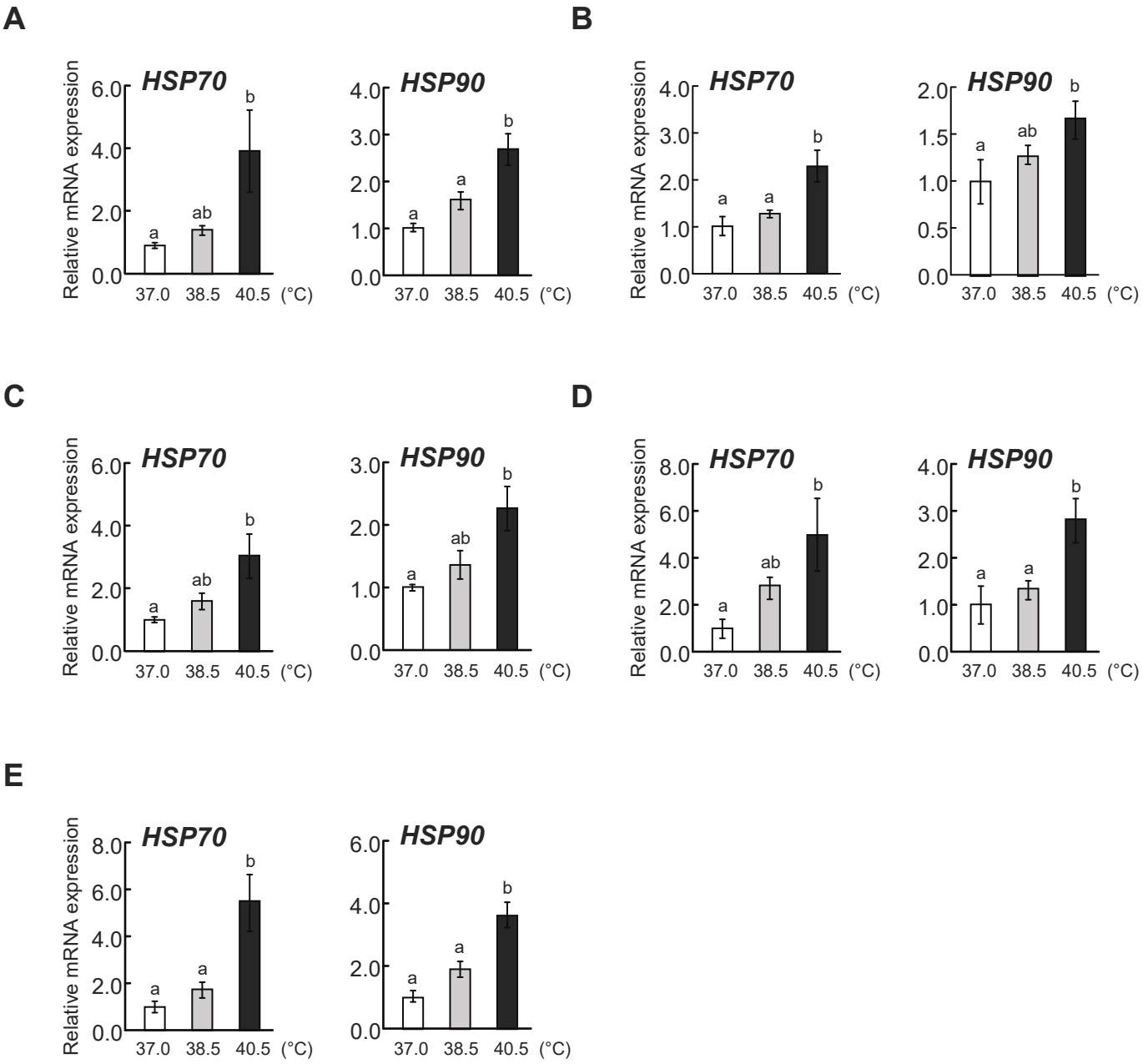

A
HSP70
Relative mRNA expression
6.0
4.0
2.0
0.0
a
ab
b
37.0
38.5
40.5
(°C)
HSP90
4.0
3.0
2.0
1.0
0.0
a
a
b
37.0
38.5
40.5
(°C)
B
HSP70
Relative mRNA expression
4.0
3.0
2.0
1.0
0.0
a
a
b
37.0
38.5
40.5
(°C)
HSP90
2.0
1.5
1.0
0.5
0.0
a
ab
b
37.0
38.5
40.5
(°C)
C
HSP70
Relative mRNA expression
6.0
4.0
2.0
0.0
a
ab
b
37.0
38.5
40.5
(°C)
HSP90
3.0
2.0
1.0
0.0
a
ab
b
37.0
38.5
40.5
(°C)
D
HSP70
Relative mRNA expression
8.0
6.0
4.0
2.0
0.0
a
ab
b
37.0
38.5
40.5
(°C)
HSP90
4.0
3.0
2.0
1.0
0.0
a
a
b
37.0
38.5
40.5
(°C)
E
HSP70
Relative mRNA expression
8.0
6.0
4.0
2.0
0.0
a
a
b
37.0
38.5
40.5
(°C)
HSP90
6.0
4.0
2.0
0.0
a
a
b
37.0
38.5
40.5
(°C)

Figure 2

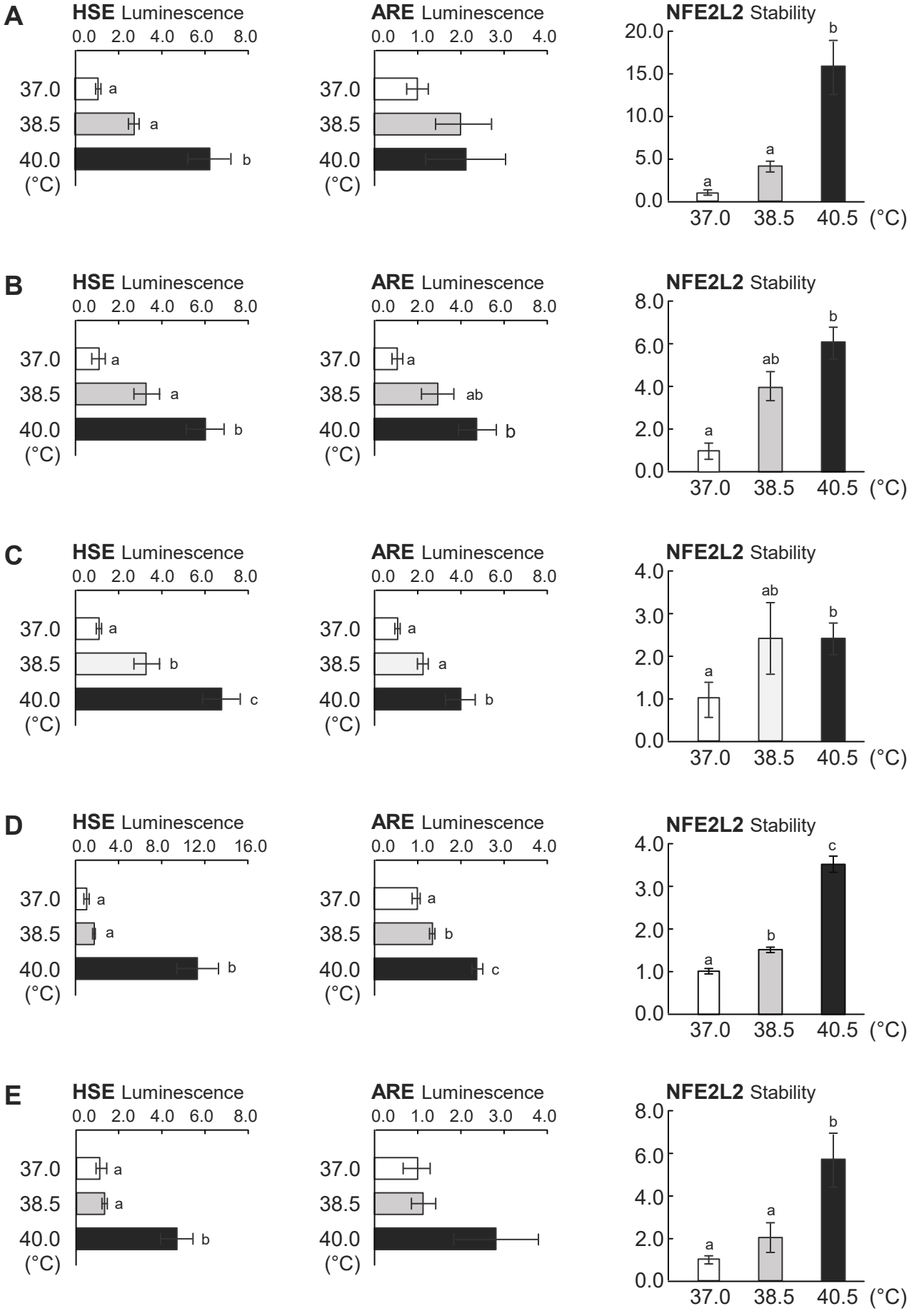

A
HSE Luminescence
0.0 2.0 4.0 6.0 8.0
37.0 a
38.5 a
40.0 b
(°C)
ARE Luminescence
0.0 1.0 2.0 3.0 4.0
37.0
38.5
40.0
(°C)
NFE2L2 Stability
20.0
15.0
10.0
5.0
0.0
a
a
b
37.0 38.5 40.5 (°C)
B
HSE Luminescence
0.0 2.0 4.0 6.0 8.0
37.0 a
38.5 a
40.0 b
(°C)
ARE Luminescence
0.0 2.0 4.0 6.0 8.0
37.0 a
38.5 ab
40.0 b
(°C)
NFE2L2 Stability
8.0
6.0
4.0
2.0
0.0
a
ab
b
37.0 38.5 40.5 (°C)
C
HSE Luminescence
0.0 2.0 4.0 6.0 8.0
37.0 a
38.5 b
40.0 c
(°C)
ARE Luminescence
0.0 2.0 4.0 6.0 8.0
37.0 a
38.5 a
40.0 b
(°C)
NFE2L2 Stability
4.0
3.0
2.0
1.0
0.0
a
ab
b
37.0 38.5 40.5 (°C)
D
HSE Luminescence
0.0 4.0 8.0 12.0 16.0
37.0 a
38.5 a
40.0 b
(°C)
ARE Luminescence
0.0 1.0 2.0 3.0 4.0
37.0 a
38.5 b
40.0 c
(°C)
NFE2L2 Stability
4.0
3.0
2.0
1.0
0.0
a
b
c
37.0 38.5 40.5 (°C)
E
HSE Luminescence
0.0 2.0 4.0 6.0 8.0
37.0 a
38.5 a
40.0 b
(°C)
ARE Luminescence
0.0 1.0 2.0 3.0 4.0
37.0
38.5
40.0
(°C)
NFE2L2 Stability
8.0
6.0
4.0
2.0
0.0
a
a
b
37.0 38.5 40.5 (°C)

Figure 3

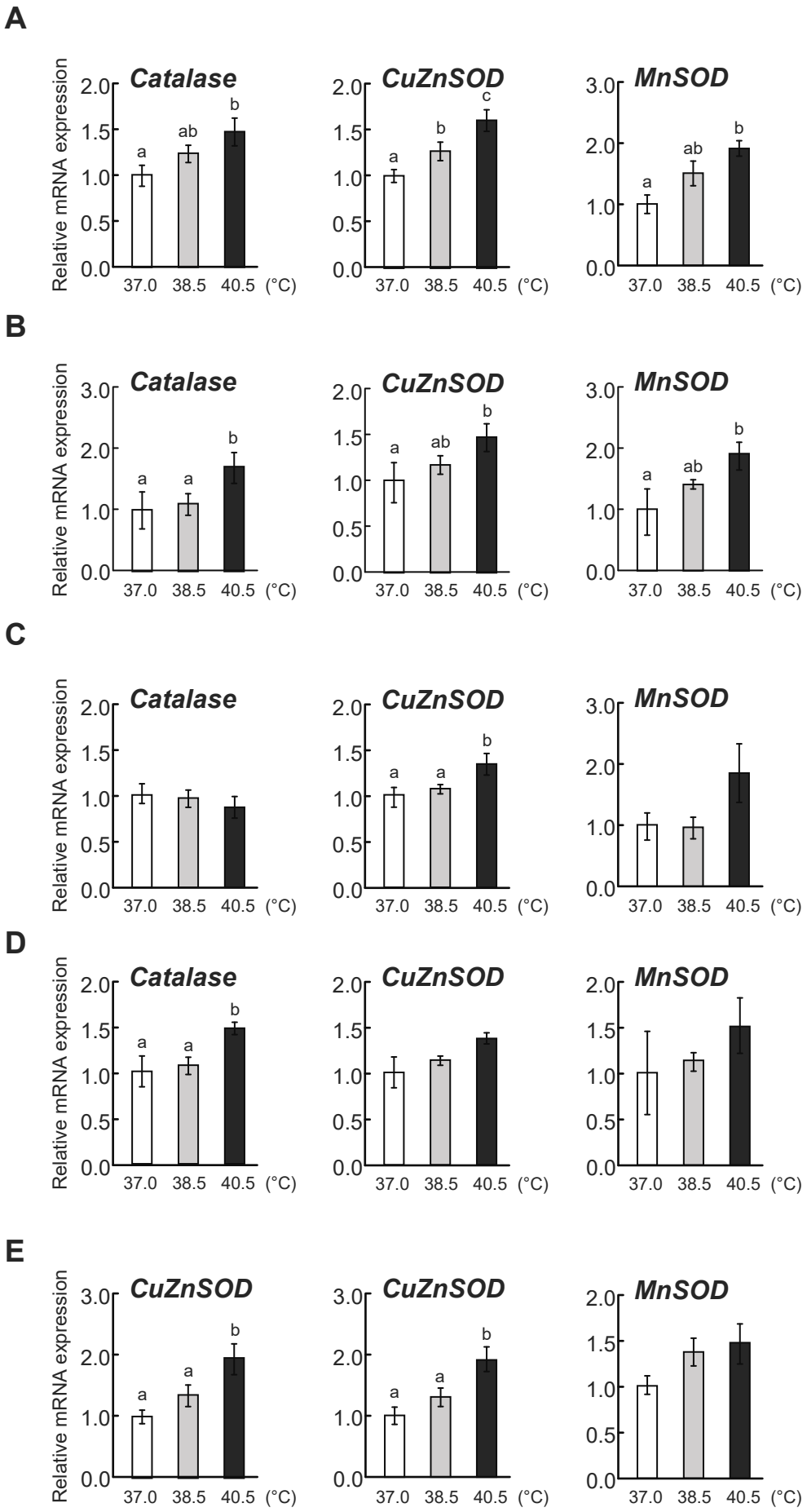

A
B
C
D
E
Catalase
CuZnSOD
MnSOD
Relative mRNA expression
37.0
38.5
40.5
(°C)
a
ab
b
c

Figure 4

A

C151

```
bKEAP1 YTASISMGEKCVLHVMNGAVM
hKEAP1 YTASISMGEKCVTHVMNGAVM
mKEAP1 YTASISVGEKCVLHVMNGAVM
```

C226

```
bKEAP1 KQEEFFNLSHCQLATLISRDD
hKEAP1 KQEEFFNLSHCQLVTLISRDD
mKEAP1 KQEEFFNLSHCQLATLISRDD
```

C273, C288

```
bKEAP1 YVQALLRAVRCHSLTPHFLQMQLQKCEILQSDSRCK
hKEAP1 YVQALLRAVRCHSLTPNFLQMQLQKCEILQSDSRCK
mKEAP1 YVQALLRAVRCHALTPRFLQTQLQKCEILQADARCK
```

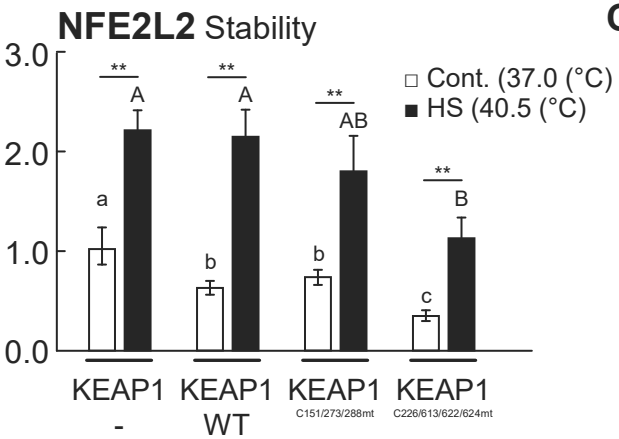
C613, C622, C624

```
bKEAP1 GVGVAVTMEPCRKQIDQQNCTC
hKEAP1 GVGVAVTMEPCRKQIDQQNCTC
mKEAP1 GVGVAVTMEPCRKQIDQQNCTC
```

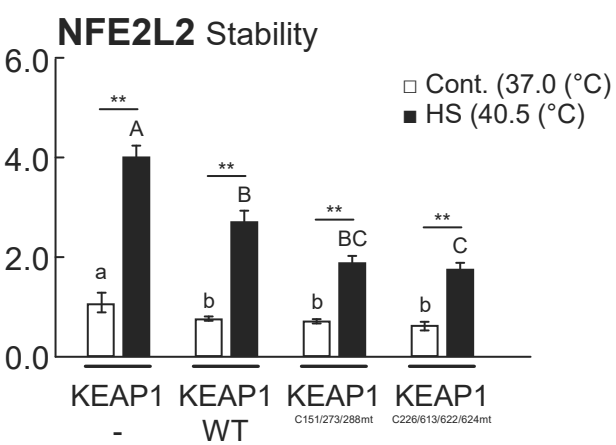
KEAP1 C151, C273, C288 mutation:
C151S (serine), C273W (tryptophan), C288E (glutamate)

KEAP1 C226, C613, C622, C624 mutation:
C226S, C613S, C622, C624 (serine)

B



C



Supple Figure 1

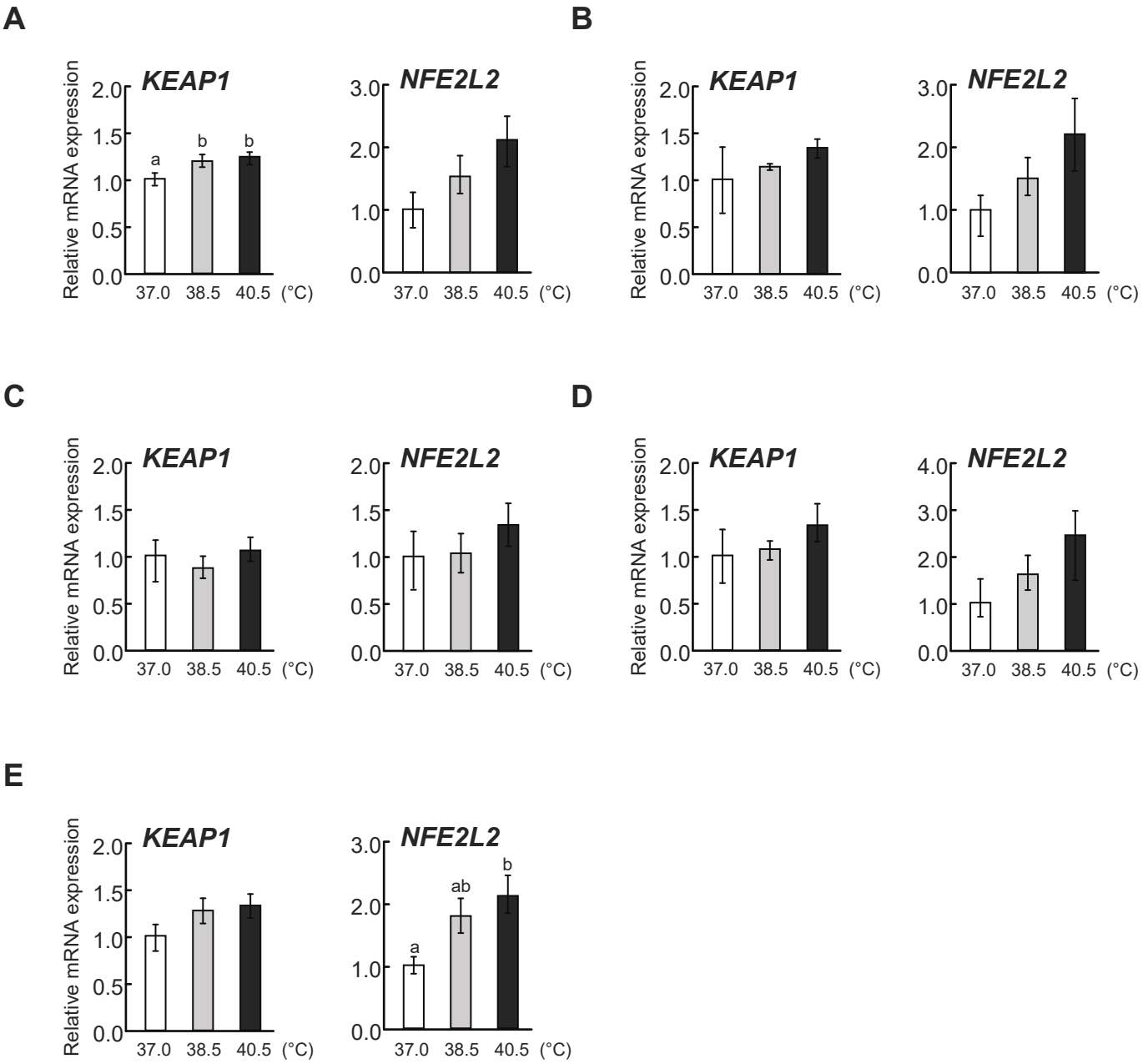


Table 1. Primers for real time PCR

Name (GenBank accession No.)	Sequence (5'-3')	Product length (bp)
Heat shock proteins		
Bovine HSP70	F: GCAGTCGGACATGAAGGAGT	109
(AY662497)	R: GATCTCCTCCGGGTAGAACG	
Homo HSP70	F: GCAGTCGGACATGAAGCACT	109
(BC002453.2)	R: GATCTCCTCGGGGTAGAACG	
Bovine HSP90	F: GGAGGATCACTTGGCTGTCA	177
(AB072368.1)	R: GGGATTAGCTCCTCGCAGTT	Park *et al*., 2021
Homo HSP90	F: GGAAGATCACTTGGCAGTGA	177
(NM_001017963.3)	R: GGGATTAGCTCCTCACAGTT	
Antioxidant enzyme genes		
Bovine Catalase	F: GGAAACGCCTGTGTGAGAAC	158
(NM_001035386.2)	R: CTGCGTTCTTAGGTTTCTCCTC	
Homo Catalase	F: GGAAACGTCTGTGTGAGAAC	157
(BC110398.1)	R: GCATTCTTAGGCTTCTCAGC	
Bovine CuZnSOD	F: ACACAAGGCTGTACCAGTGC	105
(NM_174615.2)	R: TGTCACATTGCCCAGGTCTC	Rodrigues-Cunha *et al*., 2016
Homo CuZnSOD	F: ACAGCAGGCTGTACCAGTGC	105
(EF151142.1)	R: AGTCACATTGCCCAAGTCTC	
Bovine MnSOD	F: AATCTGAGCCCTAACGGTGG	169
(NM_201527.2)	R: GTAAGCGTCCCTGCTCCTTA	Zhou *et al*., 2019
Homo MnSOD	F: AACCTCAGCCCTAACGGTGG	169
(BT006967.1)	R: GTAAGTGTCCCCGTTCCTTA	
KEAP1-NEE2L2		
Bovine KEAP1	F: ACAACAGTGTGGAGAGGTATGAGC	108
(NM_001101142.1)	R: AGAGCAGACGGTTGAGGACAG	Liang *et al*., 2019
Homo KEAP1	F: ACAACAGTGTGGAGAGGTATGAGC	108
(BC002930.1)	R: AAAGGAGACGATTGAGGACAG	
Bovine NFE2L2	F: GGACATGGATTTGATTGAC	270
(NM_001011678.2)	R: ACCTGGGAGTAGTTGGCA	Jin *et al*., 2016
Homo NFE2L2	F: GGACATGGATTTGATTGAC	270
(HM446346.1)	R: ACCTGGGAGTAGTTGGCA	
Internal control		
Bovine H2AFZ	F: AGAGCCGGTTTGCAGTTCCCG	115
(NM_174809)	R: TACTCCAGGATGGCTGCGCTGT	
Homo H2AFZ	F: GAGCCGGCTTGCAGTTCCC	115
(M37583.1)	R: TACTCCAGGATGGCTGCGCTGT	

F: Forward, R: Reverse

Table 2. Primers for reporter construction

Name	Sequence (5'-3')
Heat Shock Element	F: CTGGAAGATTCTAGAACGTTCTGGAAGATTCTAGAACGTTC
(HSE)	R: GAACGTTCTAGAATCTTCCAGAACGTTCTAGAATCTTCCAG
Antioxidant Responsive Element	F: TAGCTTGGAAATGACATTGCTAATGGTGACAAAGCAACTTTTAGCTTGGAAATGACATTGCTAATGGTGACAAAGCAACTTT
(ARE)	R: AA AGTTGCTTTGTCACCATTAGCAATGTCATTTCCAAGCTAAAAGTTGCTTTGTCACCATTAGCAATGTCATTTCCAAGCTA

F: Forward, R: Revers