The impact of acute temperature stress on hemocytes of invasive and native mussels (*Mytilus galloprovincialis* and *M. californianus*): DNA damage, membrane integrity, apoptosis and signaling pathways

Cui-Luan Yao* and George N. Somero

Hopkins Marine Station, Department of Biology, Stanford University, Pacific Grove, CA 93950 USA

*Current address: Fisheries College, Jimei University, Xiamen, 361021, People's Republic of China

*Author for correspondence (e-mail: cuiluanyao@yahoo.com)

SUMMARY

We investigated effects of acute heat- and cold stress on cell viability, lysosome membrane stability, double- and single-stranded DNA breakage, and signaling mechanisms involved in cellular homeostasis and apoptosis in hemocytes of native and invasive mussels, *Mytilus californianus* and *M. galloprovincialis*, respectively. Both heat stress (28ºC, 32ºC) and cold stress (2ºC, 6ºC) led to significant double- and single-stranded breaks in DNA. The types and extents of DNA damage were temperature- and time-dependent, as was caspase-3 activation, an indicator of apoptosis, which may occur in response to DNA damage. Hemocyte viability and lysosomal membrane stability decreased significantly under heat stress. Western blot analyses of hemocyte extracts with antibodies for proteins associated with cell signaling and stress responses [including members of the phospho-specific Mitogen Activated Protein Kinase (MAPK) family (c-JUN NH(2)-terminal kinase (JNK) and p38-MAPK) and apoptosis executor caspase-3] revealed that heat- and cold stress induced a time-dependent activation of JNK, p38-MAPK and caspase-3 and that these signaling and stress responses differed between species. Thermal limits for activation of cell signaling processes linked to repair of stress-induced damage may help determine cellular thermal tolerance limits. Our results show similarities in responses to cold- and heat stress and

29 suggest causal linkages between levels of DNA damage at both extremes of temperature and
30 downstream regulatory responses, including induction of apoptosis. Compared to *M. californianus*,
31 *M. galloprovincialis* might have a wider temperature tolerance due to a lower amount of single-
32 and double-stranded DNA damage, faster signaling activation and transduction, and stronger
33 repair ability against temperature stress.
34
35 Key words: apoptosis, cell signaling, DNA damage, hemocyte, *M. galloprovincialis*, *Mytilus*
36 *californianus*,
37 Running title: Thermal responses of *Mytilus* hemocytes

INTRODUCTION

Rocky intertidal ecosystems are commonly dominated by sessile species like mussels that encounter wide variation in abiotic conditions due to alternating periods of emersion and immersion during the tidal cycle and changes in air and water temperature due to season and latitude. Thermal stress has been shown to have pronounced influences on biogeographic and local-scale distribution patterns of many sessile species, including mussels of the genus *Mytilus* (Braby and Somero, 2006a; Jones et al., 2009, 2010). These temperature-correlated distribution patterns may be governed in large measure by the abilities of the species to cope with cellular-level damage induced by non-optimal temperatures. The emerging picture of the responses of mussels to heat stress is that rapid changes in the activities of signaling proteins, which often are a result of post-translational modifications of existing proteins (Evans and Somero, 2010) initiate diverse changes in cellular activity that function to restore cellular homeostasis. Several recent studies of congeners of *Mytilus* have shown that high temperature stress also induces changes in gene and protein expression that are indicative of damage to cellular structures and attempts by the cell to either repair this damage or, if this is not possible, to remove damaged proteins or cells from the tissue (Lockwood et al., 2010; Tomanek and Zuzow, 2010; Fields et al., 2012).

To extend the analysis of mechanisms used by congeners of *Mytilus* to cope with thermal stress and to further compare differences in stress responses between differently adapted congeners, we conducted studies that took advantage of the utility of hemocytes as an experimental system and that included a focus on cold stress as well as heat stress. Most previous studies of thermal stress in *Mytilus* have only examined effects of exposure to high temperature and have used tissues, typically gill or mantle, that contain a variety of types of cells. The work reported below exploited hemocytes, free cells in the extracellular fluids that perform a variety of functions, including immune defense, wound and shell repair, digestion, excretion, as well as transport of oxygen, nutrients and metabolites (Cheng et al., 1981; Cajaraville and Pal, 1995). Generally, there are two main populations of hemocytes, granulocytes and hyalinocytes, in mussels (Cajaraville and Pal, 1995; Carballal et al., 1997). Hemocytes afford the experimental

advantage of providing a tractable study system that is relatively well-defined in terms of cell type and where such processes as loss of membrane integrity and damage to nuclear DNA can be more readily examined with a variety of microscopic and biochemical and molecular techniques than in the case of complex tissues.

In the studies described below, we used hemocytes from two congeners of *Mytilus*, the ribbed mussel, *M. californianus*, a species native to the West Coast of North America, and the blue mussel *M. galloprovincialis*, an invasive species from the Mediterranean Sea that entered coastal California waters in the mid-20th century and has subsequently replaced the native blue mussel, *M. trossulus*, over the southern portion of its previous biogeographic range (Geller, 1999; Braby and Somero, 2006a, b; Schneider, 2008; Hilbish et al., 2010). A number of comparative studies of these three congeners of *Mytilus* have shown *M. galloprovincialis* to be more heat tolerant than the native blue mussel, and it has been conjectured that the further northward spread of the invader may be facilitated by climate change (Braby and Somero, 2006a, b; Lockwood and Somero, 2011; Fields et al., 2012). However, the effects of extreme low temperatures on congeners of *Mytilus* have received little attention, so the potential for winter conditions to influence biogeographic distributions remains largely unexplored. We thus used the hemocyte study system to examine the effects of both heat- and cold-stress on cellular status.

In our experiments we sought to characterize in hemocytes the multi-level response that is made in reaction to cellular damage, e.g., DNA breakage, from thermal stress, beginning with stress signal transduction systems and terminating with programmed cell death in cases where stress-induced damage cannot be repaired. The initial response to cellular stress commonly involves rapid activation of various signal transduction pathways that lead to either restoration of cellular homeostasis or, if this cannot be achieved, to cellular death (Kültz, 1995). p38 mitogen-activated protein kinases (MAPK), c-Jun N-terminal kinase (JNK, also called stress-activated protein kinase, SAPK) and extracellular signal-regulated kinase (ERK) constitute the family of MAPKs that commonly play key roles in the stress response. Among these systems, the p38-MAPK and JNK pathways are mainly activated by environmental stress or cytokines (Cowan and Storey, 2003). It was demonstrated that p38-MAPK and JNK activity showed a close relationship with blockage of apoptosis after thermal stress in mammalian cells lines (Brown and

Benchimol, 2006; Murai et al., 2010). Although much less is known about these responses in non-model species, p38-MAPK and JNK activation were shown to be induced by thermal stress in gill, mantle tissue or posterior adductor muscle of *M. galloprovincialis* or the bearded horse mussel *Modiolus barbatus* (Kefaloyianni et al., 2005; Anestis et al., 2007; Anestis et al., 2008; Gourgou et al., 2010).

Cellular damage from thermal stress at the molecular level has most commonly been investigated in proteins. Less understood—but of pivotal importance to the integrity of the genome—is the role of temperature-induced damage to DNA. Many previous studies have shown that DNA damage is induced by various environmental chemical stressors including genotoxic substances, heavy metals, and organic contaminants (Micic et al., 2002; Klobučar et al., 2008; Wepener et al., 2008). However, relatively little is known about effects of thermal stress on the integrity of DNA. In *M. galloprovincialis* DNA damage was detected in mantle and gill tissue following heat stress (Kefaloyianni et al., 2005), but the effects of both heat- and cold stress on different types of DNA damage remain largely unknown. DNA single-stranded breakage (SSB) is the most frequent type of DNA damage in stressed cells; SSBs are usually repaired correctly and their effects on cellular survival or mutagenesis are relatively low (Wallace, 1994). However, DNA double-stranded breakage (DSB) is far more threatening to cellular and genomic integrity and may lead to cellular death through induction of apoptosis (Ori et al., 2005). When cellular damage from stress crosses a certain threshold, notably in the case of severe damage to DNA that cannot be adequately repaired, apoptosis may be initiated. Caspase-3, well-known as the executioner caspase, plays a crucial role in apoptotic destruction of cells (Earnshaw et al., 1999; Lakhani et al., 2006). Previous studies showed that caspase-3 transcripts increased after heat stress in gill tissue (Lockwood et al., 2010) and heavy metal ion stress in mantle tissue (Kefaloyianni et al., 2005) of *M. galloprovincialis*.

Membrane systems are another critical site of damage from stress (Hochachka and Somero, 2002). Damage to lysosomes is one example of this type of stress-induced lesion. Lysosomes are found within the semi-granular and granular hemocytes of many marine invertebrates and are released by a process of degranulation of hemocytes after environmental stresses (Hauton et al., 1998; Camus et al., 2000). Once in the cytoplasm, the proteolytic enzymes that exist in lysosomes

are released and the hemocytes are lysed (Yao et al., 2008). Lysosomal neutral red retention (NRR) time has proven to be a sensitive indicator of membrane integrity of hemocytes of blue mussels and shrimp (Lowe et al., 1995; Camus et al., 2000; Yao et al., 2008). It was demonstrated that lysosome stability and membrane integrity of hemocytes have a close relationship with animal health status (Lowe et al., 1995; Yao et al., 2008). Furthermore, stress from low temperature also can reduce NRR time; this effect was observed in cold-stressed *M. galloprovincialis* (Hauton, et al., 1998; Camus et al., 2000). A recent study of thermal acclimation in the mussel *Modiolus barbatus* found decreases in NRR retention time during prolonged acclimation at high temperatures (28° and 30°C) (Dimitriadis et al., 2012).

In the present study we used hemocytes from two congeners of *Mytilus* to compare the effects of acute heat- and cold stress on double- and single-stranded DNA breakage, lysosome membrane stability, p38-MAPK and JNK phosphorylation and caspase-3 activation. Our results indicate that DNA damage, stress-related signal transduction, and apoptosis play critical roles in responses to stress from low and high temperatures by mussel hemocytes and interspecific differences in these responses may influence the thermal optima and thus the distribution ranges of the native and invasive congeners.

MATERIALS AND METHODS

Reagents

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and were of the highest grade available. The enhanced chemiluminescence (ECL) kit was from GE Healthcare (Uppsala, Sweden); the BCA protein assay reagent was from Pierce (Rockford, IL); and PVDF membranes were from Amersham (Piscataway, NJ). Antibodies specific for the phosphorylated forms of p38-MAPK (#9211) and JNKs (#9251) were obtained from Cell Signaling Technology (Beverly, MA). A rabbit monoclonal antibody specific for caspase-3 (#9665) that detects the endogenous levels pro-caspase-3 and active-caspase-3 was also purchased from Cell Signaling Technology. The anti-actin antibodies (#sc10731), HRP-conjugated anti-rabbit (#sc-2004) and anti-mouse (#sc-2055) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Prestained molecular mass standards were obtained from Bio-Rad (Hercules, CA). XOMAT AR

film was from Eastman Kodak Company (New York, NY).

Animal collections

Adult specimens of *M. galloprovincialis* (Lamarck 1819) (55-70mm length) were collected subtidally near Santa Barbara, CA (34°24′ N, 119°41′ W). Animals were shipped to Hopkins Marine Station and were maintained for four weeks at 13°C at a salinity of 31 parts per thousand in recirculating seawater tanks and fed a phytoplankton diet every day as described by Lockwood et al. (2010). *Mytilus californianus* (Conrad 1837) (55-70 mm length) were collected from mussel beds in the exposed rocky intertidal zone at Hopkins Marine Station, Pacific Grove, CA (36º37′ N, 121º54′ W) and were acclimated under the same conditions used for *M. galloprovincialis*.

Acute temperature stress

Following the acclimation period, four groups of 15 mussels of each species were acutely transferred from the 13°C holding aquaria to tanks containing either cold (2°C and 6°C) or warm (28°C and 32°C) seawater, to determine the effect of acute temperature change on survival. Survival times were based on numbers of dead animals at 8 h and 12 h after onset of acute stress. Based on survivorship at low temperatures, mussels of both species (5 individuals for each group) were immediately transferred from 13°C to 2°C and 6°C seawater aquaria for subsequent studies of cold stress. For studies of acute heat stress, *M. californianus* were acutely transferred from 13°C to 24°C, 28°C and 32°C. For the more heat-tolerant *M. galloprovincialis*, transfers were from 13°C to 28°C and 32°C. The mussels were sampled at 0.5 h, 2 h and 8 h after exposure to acute temperature stress. Mussels from the 13°C acclimation population were sampled as controls. Five mussels were sampled from each of the three treatment or control groups at each time point. Mortality during exposures was scored if mussels failed to close their shells after external stimulation. Only mussels exhibiting shell closure were used in the hemocyte experiments.

Hemocyte preparation

Hemolymph (1.2 ml per mussel) was collected from the posterior adductor muscle with a 20-gauge needle and 2 ml disposable syringe and combined with 0.3 ml of anti-coagulant solution,

a modified Alsever's solution (27 mmol l^{-1} sodium citrate, 115 mmol l^{-1} glucose, 336 mmol l^{-1} NaCl, 18 mmol l^{-1} EDTA, pH 7.0) (Li et al., 2009). Hemolymph from 5 mussels was pooled and mixed together. 600 microliters of this fresh hemolymph were used immediately to examine trypan blue exclusion, DNA integrity (comet assays), and neutral red retention by lysosomes. The rest of the hemocytes were collected by 5 min centrifugation at 1500 g, 4°C. Then, the hemocytes were resuspended in 1 ml 50 mmol l^{-1} PBS buffer (137 mmol l^{-1} NaCl, 7.8 mmol l^{-1} $Na_2HPO_4 \bullet 12H_2O$, 2.7 mmol l^{-1} KCl, 1.47 mmol l^{-1} KH_2PO_4, pH 7.4), washed twice with this buffer and collected by sedimentation. The pelleted hemocytes were then immediately frozen in liquid nitrogen and stored at -80 °C until used. The hemocytes pooled from 5 mussels at each time point were mixed as one sample; three such pooled samples were generated. Thus, a total of 15 animals were used at a time-point (n=3 measurements per pooled sample).

DNA damage assay

Double-stranded and single-stranded DNA breakage were assessed using alkaline and neutral single-cell gel electrophoresis (comet) assays, respectively. Double-stranded DNA damage was detected according to a protocol described by Singh et al. (Singh et al., 1988) with slight modifications (Klobučar et al., 2008). Briefly, 75 µl of hemolymph mixed with 0.8% low melting point (LMP) agarose were placed on a 0.75% agarose pre-coated microscope slide. After solidifying for 5 min at 4°C in the dark, a third layer of 0.5% LMP agarose was added and left to solidify as described. The cells were lysed in freshly made lysing solution (2.5 mol l^{-1} NaCl, 100 mmol l^{-1} Na_2EDTA, 10 mmol l^{-1} Tris base, 10% DMSO, 1% Triton X-100, pH 10) for 1 h at 4°C in the dark. Then, the slides were rinsed with cold alkaline electrophoresis solution for 5 min (300 mmol l^{-1} NaOH, 1 mmol l^{-1} EDTA, pH >13) and then placed on a horizontal gel box and covered with the same buffer for 20 min. The slides then were subjected to electrophoresis for 20 min at 25 V, 4°C.

Single-stranded DNA damage was assessed using the neutral comet assay. The procedure was conducted similarly to the alkaline assay but with the following lysis buffer: 2.5 mol l^{-1} NaCl, 100 mmol l^{-1} EDTA, 10 mmol l^{-1} Tris–HCl, 1% *N*-lauroylsarcosine, 0.5% Triton X-100, 10% dimethylsulphoxide (DMSO) at pH 9.5. After 1 h of lysis the slides were washed three times

with electrophoresis buffer (300 mmol l^{-1} sodium acetate, 100 mmol l^{-1} Tris–HCl, pH 8.3) and left in a fresh electrophoresis solution for 1 h. Then the slides were electrophoresed for 1 h at 14V (0.5 V/cm, 11–12 mA), 4°C.

After alkaline electrophoresis the slides were neutralized in cold neutralization buffer (0.4 mol l^{-1} Tris/HCl, pH 7.5), 2×5 min. The slides were stored in the dark at room temperature and stained with a fluorescent dye (SYBR green). The slides were stored overnight at 4°C in light-tight humidified boxes and analyses were performed the following day. For each slide, pictures of 100 randomly selected hemocytes (pooled from 5 individuals) were captured at 400 X magnification using a fluorescence microscope (Olympus). DNA damage was assessed using CASP version 1.2.2 (Comet Assay Software Project, http://casplab.com/). Triplicate analyses were done for each group. Estimates of the extent of DNA strand breakage are expressed as the percent of DNA found in the tail of the comet (%DNAT). %DNAT = (100 x DNAT)/(DNAH+DNAT). Here, DNAH (DNA head) is the sum of intensities of all points within the head of the comet and DNAT is sum of intensities of all points of the tail (Końca et al., 2003) (Fig. 1).

Cell viability

Cell viability was assessed by the trypan blue (Sigma, T8154) exclusion test, using a hemocytometer to manually count viable and nonviable cells. Briefly, 10 µl of a 0.4% solution of the dye were added to 50 µl of hemocyte suspension. The numbers of stained (blue = nonviable) and unstained (transparent = viable) hemocytes were counted using an optical microscope. For each sample (pooled hemocytes from 5 mussels), a minimum of 200 cells were counted in a total of 10 microscopic fields for each of 3 replicate preparations. Thus, a total of 9 slides representing 3 samples from 15 animals at each time point were used for analysis.

Lysosomal Stability

The stability of hemocyte lysosomes was determined using a neutral red retention protocol (Lowe and Pipe, 1994; Lowe et al., 1995). Briefly, the neutral red stock solution was made by dissolving 20 mg of dye in 1 ml of DMSO. The working solution was prepared by diluting 5 µl of stock solution in 2.5 mmol l^{-1} of PBS. Approximately 50µl of hemocyte suspension were placed

carefully on each slide. Slides then were placed in a light-proof, controlled humidity chamber for 20 min, after which the excess solution was carefully removed and 20 µl of freshly made neutral red working solution were added. The slides were incubated in the controlled humidity chamber for an additional 20 min and then were observed under an optical microscope at 400 X magnification. Tests were terminated when dye loss was evident in approximately 50% of the hemocytes.

Protein extractions

Based on initial morphological studies of hemocyte lysis, we determined temperatures of exposure and collection of hemocytes that would ensure capture of largely viable populations from both species. Because most hemocytes of *M. californianus* were lysed at 32°C, hemocytes of this species were collected at 2°C and 28°C. Hemocytes of *M. galloprovincialis* were collected at 2°C, 28°C and 32°C. Hemocytes pellets (see Hemocyte Preparation) were added to 1 volume lysis buffer (50 mmol l^{-1} Tris-HCl, pH 7.8, 250 mmol l^{-1} sucrose, 1% SDS, 0.1% NP-40) containing Complete Mini proteinase inhibitor Mix (Roche Applied Science, Indianapolis IN) (1 tablet / 10 ml). The proteins were extracted by submitting hemocytes to three 20s bursts of sonication (Branson sonicator, setting 5**)** in an ice-cold water bath. The samples were centrifuged at 12,000 x g for 10min at 4°C and the supernatants were collected. Total protein concentrations were determined using the BCA assay **(**Pierce, Rockford, IL, USA**).**

SDS-PAGE and western blotting

Thirty ng of supernatant protein were boiled with 0.33 volumes of SDS-PAGE sample buffer [0.33 mol l^{-1} Tris/HCl, pH 6.8, 10% (w/v) SDS, 13% (v/v) glycerol, 20% (v/v) 2-mercaptoethanol, 0.2% (w/v) bromophenol blue] for 3 min. The samples were loaded onto 12% (w/v) acrylamide, 0.33% (w/v) bisacrylamide Tris-HCl polyacrylamide gels. Electrophoretically separated proteins were wet transferred to PVDF membranes for 2 h at 4°C. Resulting blots were blocked for 1 h in 5% blocking grade non-fat dried milk dissolved in Tris buffered saline (250 mmol l^{-1} Tris-HCl pH 7.5, 1.5 mol l^{-1} NaCl) containing 0.1% Tween-20 (TBST), washed 2 X 5 min in TBST, and incubated with the appropriate primary antibody according to the

manufacturer's instructions.

Antibodies to detect phosphorylation on p38-MAPK (Thr-180/Tyr-182) and JNK/SAPK (Thr-183/Tyr-185), caspase-3 and actin were diluted to 1: 1000 in the same buffer. Following 3 X 5 min washes in TBST, blots were incubated with the corresponding secondary antibody (goat anti-rabbit (sc-2004) or goat anti-mouse (sc-2055) (Santa Cruz Biotechnology, Inc., Santa Cruz CA). The secondary antibody was diluted 1:3000 in 5% BSA in TBST and incubated for 60 min at room temperature with gentle agitation. Following 6 X 5 min washes in TBST, blots were treated with enhanced chemiluminescent reagent (Amersham, Piscataway, NJ) for 2 min. Finally, blots were exposed to Kodak X-Omat AR film and developed. The bands were quantified by laser scanning densitometry. Equal protein loading was verified by probing identical samples with an anti-actin antibody (whole extracts). Densitometric analyses were performed using ImageJ software (http://rsb.info.nih.gov/ij/). For the phospho-JNK/SAPK antibody, which detected two bands at 46 and 54 kDa, and for the caspase-3 antibody, which detected the endogenous levels of full-length (35 kDa) and large active fragments (17/19 kDa) of caspase-3, density was calculated as the intensity of the corresponding band. Blots and results shown are representative of three independent experiments. Results are means ± s.e. for three independent experiments.

Statistical analysis

The data were analyzed by two-way analysis of variance (two-way ANOVA) using SPSS 13.0 for Windows (SPSS Inc.). Statistical significance was determined by two-way ANOVA with stress time and temperature as factors. A least significant difference (LSD) post hoc test ($p<0.05$) was used to resolve statistically significant differences between stress temperature and stress time. Asterisks denote statistically significant differences between experimental treatments (high- and low temperature stress for varying times) and the control (13°C specimens).

RESULTS

Survival at low and high temperatures

The effects of acute cold- and heat stress on the survival of *M. californianus* and *M. galloprovincialis* were assessed by acutely exposing mussels to 2°C, 6°C, 24°C, 28°C and 32°C

and monitoring survival. For *M. californianus*, 73.3% and 20% of specimens exposed to 32°C and 28°C, respectively, died within 12 h (data not shown). 13.3% of *M. californianus* died within 8 h after exposure at 32°, but no mortality was found at 8 h after stress at 28°C. For *M. galloprovincialis* no deaths were observed after exposure at 28°C or 32°C for 8 h. For cold stress, no deaths were observed for either species following exposure to 2°C for 8 h. Thus, our results indicate interspecific differences in tolerance of high temperatures but similar tolerance of low temperatures.

DNA damage

DNA damage by environmental stress is frequently assessed by the comet assay (Singh et al., 1988). Cells with damaged DNA show increased migration of DNA fragments from the nucleus ("head" of comet) into the trailing ("tail") region of the comet; the length of the tail indicates the distribution of fragment sizes and the area of the tail provides a measure of total amount of DNA strand breakage. The most frequently used parameter for the determination of total DNA damage is the percentage of DNA in the comet's tail (Ashby et al., 1995). A representative image of the comet geometry observed in thermally stressed mussel hemocytes is shown in Fig.1.

Levels of single-stranded DNA damage in hemocytes are shown in Fig. 2. In hemocytes of *M. californianus* at the control temperature (13°C), DNA with single-stranded breakage (SSB) represented about 30% of total DNA (Fig. 2A). SSBs increased significantly (approximately doubling) after 8 h stress at 2°C and 6°C ($p<0.05$). Significant increases of SSBs occurred more rapidly under heat stress and were detected after 0.5 and 2 h exposure at 32°C ($p<0.05$). However, after 8 h at 32°C, the level of SSBs did not differ from control values.

The levels of SSBs in *M. galloprovincialis* hemocytes following different temperature stresses are shown in Fig. 2B. In control hemocytes, a lower fraction of the DNA was found in the tail of comet, approximately 5%, than in the case of *M. californianus*. However, as in the latter species, SSBs in hemocytes of *M. galloprovincialis* increased under both low- and high temperature stress, with the greatest tail DNA value, 50.3%, found after 8 h exposure at 32°C ($p<0.05$).

The extent of double-stranded DNA breakage (DSB) in hemocytes under control conditions (13°C) (Fig. 3) was much less than the extent of SSB (Fig. 2). Distinct interspecific differences were noted, with damage in hemocytes of *M. californianus* being greater than in hemocytes of *M. galloprovincialis* at the corresponding time and stress temperature (Fig. 3). In *M. californianus* hemocytes, DSBs increased gradually from the beginning of thermal stress and reached a peak value after 8 h exposure at 2°C, 6°C, 24°C and 28°C (Fig. 3A). At 32°C, however, DSBs increased sharply after 0.5 h exposure and the high value was maintained to 8 h, with approximately 95% of the DNA occurring in the tail region of the comet. In *M. galloprovincialis* hemocytes, DSBs showed a gradual increase after cold- or heat stress and did not reach values as high as those found for its congener (note the different ordinate ranges in Fig.3A and B). The most serious damage appeared at 8 h after cold- and heat stresses ($p<0.05$), with the peak value of approximate 50% tail DNA at 8 h after stress at 32°C (Fig. 3B).

In summary, hemocytes of the two congeners exhibited large differences in SSBs and DSBs under most conditions. SSB levels were generally higher in *M. californianus* hemocytes, including under control conditions. DSB levels were temperature- and time-dependent in both species after cold and heat stress. As in the case of SSBs, higher levels of DSBs were found in *M. californianus* hemocytes under most experimental conditions. These data suggest that thermal stress has significant effects on the integrity of DNA and that this stress varies between congeners. Thus, in the case of DSBs, *M. californianus* hemocytes attained extremely high levels of DSBs at elevated temperature (32°C), levels that were approximately twice those observed in *M. galloprovincialis.*

Stress response: p38-MAPK and JNK/SAPK activation

For *M. californianus,* p38 phosphorylation level increased significantly after 2 h of cold stress at 2°C, with the highest expression reaching 1.8 times that of the control group ($p<0.05$) (Fig. 4A). The phosphorylation level then gradually decreased, but a moderately high expression of phospho-p38 was maintained to 8 h, although this level was not significantly different from the control. Exposure to 28°C did not lead to significant changes in phospho-p38 levels. For *M. galloprovincialis*, phospho-p38 increased significantly from 0.5 h to 2 h ($p<0.05$) after cold stress

at 2°C, and then returned to the control level at 8 h (Fig. 4B). After high temperature stress at 28°C, phospho-p38 increased gradually with exposure up to 8 h, reaching a peak value 4 times that of the control group ($p<0.05$). At 32°C, phospho-p38 increased significantly at 0.5 h ($p<0.05$), returned to control levels at 2 h, and then increased again from 2 to 8 h of stress ($p<0.05$) (Fig. 4B).

Expression profiles of phospho-JNK (Thr-183/Tyr-185) (46 kDa bands for phospho-JNK1, 54 kDa bands for phospho-JNK2/3) at different temperature stresses are shown in Fig. 4C-F. In *M. californianus*, the expression level of phospho-JNK1 (46 kDa) decreased sharply at 0.5 h after cold stress at 2°C ($p<0.05$), however, it returned to control levels between 2 to 8 h. After heat stress at 28°C, phospho-JNK1 showed a significant decrease at 2 h ($p<0.05$), and then recovered to that of the control level at 8 h (Fig. 4C). In *M. galloprovincialis*, phospho-JNK1 (46 kDa) showed a gradual increase after cold stress at 2°C, with the highest level appearing at 8 h ($p<0.05$) (Fig. 4D). After heat stress, phospho-JNK1 (46 kDa) increased gradually from 0.5 to 8 h at 28°C ($p<0.05$), reaching a peak value of 3.2 times the control level at 8 h after heat stress at 28°C. The phospho-JNK1 (46 kDa) level showed a significant increase from 0.5 and 8 h at 32°C ($p<0.05$) (Fig. 4D). Phospho-JNK2/3 in *M. californianus* (54 kDa) also dropped sharply at 0.5 h under 2°C exposure ($p<0.05$); then it recovered moderately at 2 h but decreased again at 8 h ($p<0.05$). Phospho-JNK2/3 (54 kDa) showed similar changes to JNK1 after heat stress at 28°C (Fig. 4E). In *M. galloprovincialis*, Phospho-JNK2/3 (54 kDa) also increased significantly at 0.5 and 8 h ($p<0.05$) with the peak value 5.3 times that of the control group ($p<0.05$) at 8 h after 2°C stress (Fig. 4F). After heat stress at 28°C, phospho-JNK2/3 (54 kDa) level increased sharply at 0.5 h ($p<0.05$) and then gradually returned to the control level at 8 h. A similar change of phospho-JNK2/3 was found after heat stress at 32°C; phosphorylation level of JNK2/3 increased significantly at 0.5 h ($p<0.05$) and then decreased gradually from 0.5 to 8 h after stress at 32°C, with the significant decrease expression appeared at 8 h ($p<0.05$) (Fig. 4F).

Overall, our results showed that the phosphorylation level of p38 and JNKs in *M. galloprovincialis* hemocytes after cold and heat stress increased faster and reached high levels than in the case of *M. californianus*.

386 **Apoptosis initiation: caspase-3 activation**

387 The caspase-mediated apoptotic death induced by diverse stressful conditions is well
388 characterized in mammalian cell types (for a review, see Jiang and Wang, 2004). Expression
389 profiles of pro- and active-caspase-3 in hemocytes after cold- and heat stress are shown in Fig.5.
390 In *M. californianus*, pro-caspase-3 expression increased significantly at 0.5 h after cold stress at
391 2°C, and then decreased gradually even though a significantly higher level of pro-caspase-3
392 expression was maintained out to 8 h of exposure ($p<0.05$). At 28°C, pro-caspase-3 expression
393 showed a similar change with that of cold stress ($p<0.05$) (Fig. 5A). In *M. galloprovincialis*,
394 pro-caspase-3 expression showed significant increase at 0.5 h after cold stress at 2°C, after which
395 it decreased gradually and returned to the control level at 8 h (Fig. 5B). At 28°C, pro-caspase-3
396 was up-regulated significantly from 0.5 h to 8 h after stress, with the peak value at 8 h of 3.15
397 times that of the control group. Pro-caspase-3 also increased significantly from 0.5 to 8 h after
398 heat stress at 32°C ($p<0.05$), but the highest level was lower than the group under 28°C stress (Fig.
399 5B).

400 Active-caspase-3 expression in *M. californianus* increased sharply at 0.5 h ($p<0.05$) after
401 cold stress at 2°C; subsequently, it decreased although significantly higher expression lasted to 8 h
402 after stress ($p<0.05$) (Fig. 5C). At 28°C, active-caspase-3 expression levels showed a gradual
403 increase from 0.5 to 8 h after stress, with significantly higher expression levels occurring at 2 and
404 8 h ($p<0.05$) (Fig. 5C). In *M. galloprovincialis*, active-caspase-3 showed significant enhancement
405 at 0.5 h after cold stress at 2°C, but decreased gradually and returned to the control level at 8 h
406 (Fig. 5D). At 28°C active-caspase-3 expression level increased gradually from 0.5 h to 8 h
407 ($p<0.05$), with the peak value appearing at 8 h after stress. At 32°C, significantly higher
408 expression of active-caspase-3 was found at 0.5 and 2 h ($p<0.05$); expression retuned to the
409 control level at 8 h of stress (Fig. 5D).

410 In summary, caspase-3 expression could be induced by both cold and heat stress in the two
411 species. However, the species differed in their responses in terms of total levels of the protein and
412 the time-courses of the response. For example, after exposure to 28°C the highest pro-caspase-3
413 level was detected at 0.5 h in *M. californianus* hemocytes whereas the greatest pro-caspase-3
414 expression in *M. galloprovincialis* was found at 8 h.

Temperature stress causes necrosis and lysis of hemocytes

Hemocyte viability was assessed using the trypan blue exclusion test. At the control temperature of 13°C, fewer than 5% of *M. californianus* hemocytes were non-viable (Fig. 6A). Cold- and heat stress led to significant increases in percentages of nonviable cells. The hemocytes of *M. californianus* were ~10% non-viable after 0.5 h of cold stress at 2°C, and the fraction of non-viable hemocytes increased to 27% at 2 h and 22.7 % at 8 h after stress at 2°C. At 6°C, the viability of hemocytes showed similar changes to those found with the 2°C stress group. At 24°C, percentage of non-viable hemocytes showed a gradual increase from 0.5 to 8 h. Under 32°C stress, the percentage of non-viable hemocyte gradually increased with time of stress, with a peak value of 40% at 8 h ($p<0.05$).

Hemocyte viability for *M. galloprovincialis* is shown in Fig. 6B. Under control (13°C) conditions, hemocyte viability was near 98% at all time points. After cold stress at 2°C, the non-viable hemocytes showed a gradual increase from 0.5 h to 8 h, with the highest value of 15.7 % non-viable hemocytes appearing at 8 h. There was no significant difference between cold stress at 2°C and at 6°C. After heat stress at 28°C, hemocyte non-viability showed a gradual increase from 0.5 h to 8 h. At 32°C, hemocyte viability showed a similar change to that seen in the specimens exposed to 28°C, with the greatest percentage of non-viable hemocytes reaching 34% ($p<0.05$).

Stability of lysosomal membranes in hemocytes was assessed using the neutral red retention assay (Fig. 7). Both cold- and heat stress reduced neutral red retention times (NRRT) significantly. In *M. californianus*, the NRRT was ~ 109 min at 13°C. After cold stress at 2°C, the NRRT decreased to 60 min at 0.5 h and continued decreasing from 0.5 to 8h, with the shortest NRRT of ~30 min at 8 h ($p<0.05$). The NRRT after cold stress at 6°C showed a similar pattern to that seen in the group under 2°C stress. Additionally, the NRRT decreased significantly after stress at 24°C, 28°C and 32°C. NRRT showed time- and temperature-dependent patterns, with the shortest NRRT of ~10 min at 8 h after heat stress at 32°C (Fig. 7A).

In *M. galloprovincialis*, the NRRT was >120 min in the control group (13°C). During exposure to 2°C NRRT showed a gradual decrease from 0.5 to 8 h; the shortest NRRT (~70 min)

occurred at 8 h. At 6°C, a similar pattern to that seen at 2°C was observed; however, the NRRT was longer than that of the 2°C specimens at corresponding time points. After stress at 28°C, NRRT was ~100 min at 0.5 h; it then decreased gradually and reached a minimal value of ~59 min at 8 h. At 32°C, NRRT decreased sharply, with the smallest value of ~30 min occurring at 8 h of stress exposure ($p<0.05$) (Fig. 7B).

Overall, although both species showed qualitatively similar responses to thermal stress in viability and NRRT, the percentage of non-viable hemocytes typically was higher and the NRR time generally was shorter in *M. californianus* than in *M. galloprovincialis* at the corresponding temperature and time point. Differences after 2 hours of cold stress were especially marked between species.

DISCUSSION

Whole organism thermal tolerance

After acute high temperature stress, *M. californianus* showed a much higher mortality (73.3% at 32°C for 12 h) and a lower high temperature limit (28°C for 8 h) than *M. galloprovincialis*, which survives at 32°C for 8 h. These findings agree with those of previous studies that demonstrated that *M. galloprovincialis* is a warm-adapted species relative to its congeners (Braby and Somero, 2006b; Fields et al., 2006; Lockwood and Somero, 2011; Fields et al., 2012). *M. californianus* and *M. galloprovincialis* could survive for 8 h at 2°C, suggesting that both species may be tolerant of cold extremes at least over short time intervals. However, their mortality and tolerance ability after long term exposure need further study.

DNA damage

DNA is vulnerable to damage from a variety of toxic insults, including those that result from normal metabolic activities, e.g., the production of reactive oxygen species, and from physical and chemical environmental stressors. DNA damage due to combinations of environmental factors and normal metabolic processes occurs at a high rate in human cells and increases under environmental stress (Roberts et al., 2006; Prasad et al., 2011). Single base lesions

causing single-stranded breakage are the most common forms of DNA damage (Roberts et al., 2006; Prasad et al., 2011). Although SSBs can generally be repaired, they are known to be the initial signal for activating the SOS repair response in bacteria (Craig and Roberts, 1981) and to act as the initial signal for DNA damage responses in eukaryotic cells (Li and Deshaies, 1993). A number of studies have demonstrated that production of SSBs can influence the cell cycle and induce cell death, indicating a broad potential role of single-stranded DNA in DNA damage signaling.

The amounts of SSB and DSB in hemocytes from mussels subjected to heat- and cold stresses varied between species and with temperature and time of exposure to stress. In the case of SSBs, in hemocytes of *M. californianus* at the control temperature of 13°C, a typical seawater temperature in Monterey Bay, approximately 30% of the total DNA was in the tail of the comet, indicating a substantial number of SSBs even in the absence of thermal stress. In contrast, in hemocytes of *M. galloprovincialis* only ~5-10% of the DNA reflected SSBs at 13°C (Fig. 2). Cold stress (2°C and 6°C) led to increased SSB in both species, but a higher level of SSB again occurred in *M. californianus*. Heat stress at 28°C and 32°C also increased levels of SSBs. Similar amounts of SSBs were seen in the two congeners under heat stress.

Double-stranded breaks are thought to be the most serious form of DNA damage because they can impede transcription, replication, and chromosome segregation (Nitiss, 1998). The percentage of DSBs was low in hemocytes of both species at 13°C (Fig. 3). Cold stress and heat stress both led to significant increases in DSBs, with *M. californianus* showing a higher level of DSBs than its congener under all conditions of stress. Thus, at 32°C, 8 h of exposure led to approximately 100% DSBs in hemocytes in *M. californianus*, whereas this stress exposure led to only about 50% DSBs in *M. galloprovincialis*. The interspecific differences in DSBs under cold and heat stress suggest a greater thermal tolerance range for DNA stability in *M. galloprovincialis* relative to *M. californianus*. In accord with interspecific differences among congeners of *Mytilus* in other physiological traits (Lockwood and Somero, 2011), the higher resistance of *M. galloprovincialis* to thermally induced damage to DNA, notably in DSB levels, could be important in conferring on this species its capacity to invade a variety of habitats.

The findings of significant and species-specific damage to DNA under cold- and heat

stress indicate that the role of damage to DNA under different environmental conditions merits further study in the contexts of energetic costs for repair, for example in cases where severe DNA damage leads to apoptosis, and potential compromise of the integrity of the genome.

Stress signaling proteins

Recently, studies have demonstrated that p38-MAPK and JNK play crucial roles in the adaptive responses to thermal, osmotic, oxidative and heavy metal stresses in mussels (Kefaloyianni et al., 2005; Gaitanaki et al., 2007; Evans and Somero, 2010; Gourgou et al., 2010). For example, hyperthermia (30ºC), hypothermia (4ºC) and heavy metals induced a significant activation of p38-MAPK in gill and mantle tissue of *M. galloprovincialis* (Kefaloyianni et al., 2005). Significant p38-MAPK activation was detected in *M. galloprovincialis* mantle tissue after oxidative and hypertonic stress (Gaitanaki et al., 2004), and in *M. galloprovincialis* and *Modiolus barbatus* mantle tissue after high temperature stress (Anestis et al., 2007; Anestis et al., 2008).

Significant increase of phospho-p38-MAPK was detected in hemocytes of *M. californianus* and *M. galloprovincialis* after cold stress at 2°C, suggesting that phosphorylation of p38-MAPK played an important role in response to cold stress in both species. Gaitanaki and colleagues (Gaitanaki et al., 2004) demonstrated that cold temperature stress (4°C) induced a moderate phospho-p38-MAPK response in mantle tissue of *M. galloprovincialis*. Our results showed that phospho-p38-MAPK level increased significantly in hemocytes of *M. galloprovincialis* at 28°C; however, it did not show significant change in hemocytes of *M. californianus* under this condition. Similarly, exposure to high temperature was found to cause a significant and sustained stimulation of p38-MAPK phosphorylation in the gill tissue of *M. galloprovincialis* (Gourgou et al., 2010; Kefaloyianni et al., 2005). Our results indicate that *M. californianus* might lose this component of the cellular stress response at temperatures of 28°C and higher, whereas *M. galloprovincialis* retains this ability. This cellular-level difference in thermal limits of signaling ability might be one of the factors that contribute to the higher heat tolerance of *M. galloprovincialis* (Fig. 4B).

Evans and Somero (Evans and Somero, 2010) demonstrated that up-regulation of phospho-JNK/SAPK increased significantly in gill of *M. galloprovincialis* after heat stress at 32°C while it decreased significantly by 24°C in *M. californianus*. Gill tissue of *M. galloprovincialis*

(Gourgou et al., 2010) and mantle tissue and posterior adductor muscle of both *M. galloprovincialis* and *M. barbatus* also showed activation of JNK at high temperature (Anestis et al., 2007; Anestis et al., 2008), suggesting a common cellular stress response to heat stress in different cell types of these two mussels. Results of the present study showed that both cold- and heat stress induced a significant increase in JNK phosphorylation in hemocytes of *M. galloprovincialis*, with the strongest expression at 28°C. However, compared with p38-MAPK, JNK/SAPK activation was relatively moderate. In contrast, in *M. californianus* JNK phosphorylation showed a significant decrease after cold stress at 2°C and heat stress at 28°C, suggesting that a stronger response of phosphorylation of p38-MAPK and JNK to the low- and high temperature stress existed in hemocytes of the invasive species *M. galloprovincialis*.

The different response of p38-MAPK and JNKs might be due to their different roles in hemocytes in the face of temperature stress. Exposure of *M. galloprovincialis* to 30°C was found to cause a significant and sustained stimulation of p38-MAPK phosphorylation while the activation profile of JNKs was transient and relatively moderate in gill tissue (Gourgou et al., 2010; Evans and Somero, 2010). It was demonstrated that the p38-MAPK phosphorylation was activated more rapidly and strongly than that of JNKs in the isolated perfused heart of the frog *Rana ridibunda* at 42°C (Gaitanaki et al., 2008), further indicating that p38 and JNKs might play different roles in animals during temperature stress.

Because stress-induced kinases play a number of different roles, ranging from modulating repair mechanisms to directing cells to apoptosis, and because they interact among themselves in complex fashions to shape the overall responses of cells to stress, it is not possible to discern from the present data how the species differ in the ultimate cellular responses governed by these molecules. Thus, study needs to be extended to elucidate adequately the downstream consequences of changes in the abundances and post-translational modification states of these signaling proteins, e.g., whether or not apoptosis is triggered.

Caspase-3 activation

Caspase-3 is one of the key enzymes in apoptotic destruction of cells and is called the executioner caspase (Earnshaw et al., 1999). Caspase-3 expression increased significantly in hemocytes of

both species after heat stress, suggesting that single- and double-stranded DNA damage could not be adequately repaired in either species under these extremes of temperature (Fig. 5) and that apoptosis may have been the end-result of this damage.

Note that in hemocytes of *M. galloprovincialis* exposed to 32°C, pro-caspase-3 and active caspase-3 levels were lower than those measured in 28°C-exposed specimens (Fig. 5B,D). In a study of transcriptional responses to heat stress, caspase-3 transcripts increased after heat stress in gill tissue of *M. galloprovincialis* after exposure to 28°C, but decreased after exposure at 32°C (Lockwood et al., 2010). The lower levels of caspase-3 mRNA and pro- and active-caspase-3 observed at 32°C likely reflect thermal disruption of caspase synthesis and post-translational processing, not a reduced level of DNA damage at 32°C. In fact, DSB damage was higher at 32°C than at 28°C (Fig. 3B). Thus, near 32°C, a key component of the regulatory network needed for completing the cellular stress response may be disabled in this species. Thermal limits for essential components of the cellular stress response may be instrumental in establishing whole organism lethal temperature ranges.

At 2°C both species exhibited increased active-caspase-3 expression (Fig. 5), which reflected the general pattern of DSB observed at low temperature (Fig. 3). Active caspase-3 levels remained above control (13°C) levels in *M. californianus* out to 8 h of exposure to cold, but those of *M. galloprovincialis* decreased to control values by 8 h. These interspecific differences may reflect variations in levels of DNA damage and in DNA repair capacity, as discussed above.

Hemocyte viability and lysosome integrity

Hemocyte viability generally decreased with exposure time during low- and high-temperature stress in both species (Fig. 6). The species responded similarly to exposure to 28°C and 32°C, but *M. galloprovincialis* was slightly less sensitive to heat stress. There was no difference between the congeners in hemocyte viability during acute cold exposure.

Stability of lysosomal membranes as indexed by NRRT also decreased in a time-dependent manner under cold- and heat stress (Fig. 7). NRRT generally reached lower values in *M. californianus*, suggesting lower membrane stability in this species. Reductions in hemocyte viability and NRRT appear to be generally useful indices of cellular stress. Previous studies also

have demonstrated that hemocyte viability and lysosome membrane stability decreased when the animal suffered from environmental stress or pathogenic infection (Hauton et al., 1998; Camus et al., 2000; Yao et al., 2008; Parolini et al., 2011).

Conclusions

The results of the present study demonstrate the utility of hemocytes as an experimental system for examining a number of aspects of cellular thermal stress in marine mollusks and other taxa that possess these types of cells. Stress from low- and high temperatures led to increased DNA damage in hemocytes of *M. californianus* and *M. galloprovincialis*, notably in the case of DSBs. DNA damage may be important per se, due to effects on genome integrity, as well as serving as a critical factor in downstream initiation of apoptosis. The finding that stress from low temperatures (2°C and 6°C) led to levels of DSB similar to and, at times, higher than the DSB levels found under heat (28°C) stress suggests that more extensive analysis of cold stress—which commonly has received less attention than heat stress—is warranted. This conclusion is supported as well by the findings that cellular viability and lysosomal membrane integrity are also compromised at low, as well as at high temperatures. Likewise, and in concert with the findings of DNA damage at low and high extremes of temperature, the finding that caspase-3 levels increased under exposure to low as well as high temperatures indicates that cold- and heat stress can be sufficient to trigger programmed cell death (apoptosis).

Our results also shed light on the diverse cell-signaling processes that are involved in responding to cold- and heat stress and how these responses may differ between species. The activation by heat stress of key regulatory proteins, for example, phosphorylation on p38-MAPK and JNK, might play important roles in inducing down-stream molecular responses to stress, such as regulating caspase-3 function. Thermal limits to initiation of stress-signaling processes, as discovered in this study, may contribute to establishing whole organism tolerance limits. The temperature ranges over which the cellular stress response can be activated may play important roles in governing the thermal tolerance of the whole organism.

Lastly, our data provide additional insights into evolved differences in thermal tolerances between these two congeners of *Mytilus*. Relative to *M. californianus*, *M. galloprovincialis* appears, by

several criteria, to be more robust in the face of thermal stress. Thus, the invasive species exhibited a lower amount of single- and double-stranded DNA damage and greater hemocyte membrane stability against cold- and heat stress. In addition, activation of p38-MAPK and JNK was greater in the invasive species than in the native species. Whereas the initiation of a strong stress response might be interpreted as a reflection of a high level of damage to cells, it could alternatively be an indication of a relative high capacity for dealing with such damage. Thus, a capacity to initiate a stronger stress response and to do so rapidly might be a mechanistic basis for greater tolerance in stress-resistant species. Further comparative studies of the downstream consequences of early cell-signaling events are clearly warranted. The physiological differences found in past studies and the present investigation may provide at least a partial explanation for this invasive species' ability to enter and thrive in habitats at numerous sites around the globe.

ACKNOWLEDEMENTS

We would like to thank Dr. Xiande Liu (Jimei University) for help with the statistical analysis.

This research was funded by National Science Foundation grant IOS-0718734 to GNS and National Science Foundation grant of China 41076076 to CLY.

REFERENCES

Anestis, A., Lazou, A., Pörtner, H.O. and Michaelidis, B. (2007). Behavioral, metabolic, and molecular stress responses of marine bivalve *Mytilus galloprovincialis* during long-term acclimation at increasing ambient temperature. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **293**, R911-R921.

Anestis, A., Pörtner, H.O., Lazou, A. and Michaelidis, B. (2008). Metabolic and molecular stress responses of sublittoral bearded horse mussel *Modiolus barbatus* to warming sea water: implications for vertical zonation. *J. Exp. Biol.* **211**, 2889-2898.

Ashby, J., Tinwell, H., Lefevre, P.A. and Browne, M.A. (1995). The single cell gel electrophoresis assay for induced DNA damage (comet assay): measurement of tail length and moment. *Mutagenesis*. **10**, 85-90.

Braby, C.E. and Somero, G.N. (2006a). Ecological gradients and relative abundances of native

(*Mytilus trossulus*) and invasive (*Mytilus galloprovincialis*) blue mussels in the California hybrid zone. *Mar. Biol.* **148**, 1249-1262.

Braby, C.E. and Somero G.N. (2006b). Following the heart: temperature and salinity effects on heart rate in native and invasive species of blue mussels (genus *Mytilus*). *J. Exp. Biol.* **209**, 2554-2566.

Brown, L. and Benchimol, S. (2006). The involvement of MAPK signaling pathways in determining the cellular response to p53 activation: cell cycle arrest of apoptosis. *J. Biol. Chem.* **281**, 3832–3840

Cajaraville, P.M. and Pal, S.G. (1995). Morphofunctional study of the haemocytes of the bivalve mollusc *Mytilus galloprovincialis* with emphasis on the endolysosomal compartment. *Cell Struct. Funct*. **20**, 355-367.

Camus, L., Grùsvik, B.E., Bùrseth, J.F., Jones, M.B. and Depledge, M.H. (2000). Stability of lysosomal and cell membranes in haemocytes of the common mussel (*Mytilus edulis*): effect of low temperatures. *Mar. Environ. Res*. **50**, 325-329.

Carballal, M.J., Lopez, M.C., Azevedo, C. and Villalbal, A. (1997). Hemolymph cell types of the mussel *Mytilus galloprovincialis*. *Dis. Aquat. Org*. **29**, 127-135.

Cheng, T. C. (1981). Bivalves. In *Invertebrates Blood Cells* (ed. N. A. Ratcliffe and A. F. Rowley), pp. 233-300. London: Academic Press.

Cowan, K.J. and Storey, K.B. (2003). Mitogen-activated protein kinases: new signaling pathways functioning in cellular responses to environmental stress. *J. Exp. Biol.* **206**, 1107-1115.

Craig, N. L., and Roberts, J. W. (1981) Function of nucleoside triphosphate and polynucleotide in Escherichia coli recA protein-directed cleavage of phage lambda repressor. *J. Biol. Chem.* **256**, 8039–8044.

Dimitriadis, V.K., Gougoula, C., Anestis, A., Pörtner, H.O. and Michaelidis, B. (2012). Monitoring the biochemical and cellular responses of marine bivalves during thermal stress by using biomarkers. *Mar. Environ. Res.* **73**, 70-77.

Earnshaw, W. C., Martins, L. M. and Kaufmann, S. H. (1999). Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annu. Rev. Biochem*. **68,** 383-424.

Evans, T.G. and Somero, G.N. (2010). Phosphorylation events catalyzed by major cell signaling proteins differ in response to thermal and osmotic stress among native (*Mytilus californianus* and *Mytilus trossulus*) and invasive (*Mytilus galloprovincialis*) species of mussels. *Physiol. Biochem. Zool.* **83,** 984-996.

Fields, P.A., Rudomin, E.L. and Somero, G.N. (2006). Temperature sensitivities of cytosolic malate dehydrogenases from native and invasive species of marine mussels (genus *Mytilus*): sequence-function linkages and correlations with biogeographic distribution. *J. Exp. Biol.* **209**, 656-667.

Fields, P.A., Zuzow, M. and Tomanek, L. (2012). Proteomic responses of blue mussel (*Mytilus*) congeners to temperature acclimation. *J. Exp. Biol.* **215**, 1106-1116.

Gaitanaki, C., Kefaloyianni, E., Marmari, A. and Beis, I. (2004). Various stressors rapidly activate the p38-MAPK signaling pathway in *Mytilus galloprovincialis* (Lam.) *Mol. Cell. Biochem.* **260**, 119-127.

Gaitanaki, C., Mastri, M., Aggeli, I.K.S. and Beis, I. (2008). Differential roles of p38-MAPK and JNKs in mediating early protection or apoptosis in the hyperthermic perfused amphibian heart. *J. Exp. Biol*. **211**, 2524-2532.

Gaitanaki, C., Pliatska, M., Stathopoulou, K. and Beis, I. (2007). Cu^{2+} and acute thermal stress induce protective events via the p38-MAPK signalling pathway in the perfused *Rana ridibunda* heart. *J. Exp. Biol*. **210**, 438-446.

Geller, J. B. 1999. Decline of a native mussel masked by sibling species invasion. *Conserv. Biol*. **13**, 661 – 664.

Gourgou, E., Aggeli, I.K., Beis, I. and Gaitanaki, C. (2010). Hyperthermia-induced Hsp70 and MT20 transcriptional upregulation are mediated by p38-MAPK and JNKs in *Mytilus galloprovincialis* (Lamarck); a pro-survival response. *J. Exp. Biol*. **213**, 347-357.

Hauton, C., Hawkins, L.E. and Hutchinson, S. (1998). The use of the neutral red retention assay to examine the effects of temperature and salinity on haemocytes of the European flat oyster *Ostrea edulis* (L). *Comp. Biochem. Physiol*. *B* **119**, 619–623.

Hilbish, T., Brannock, P., Jones, K., Smith, A., Bullock, B. and Wethey, D. (2010). Historical changes in the distributions of invasive and endemic marine invertebrates are contrary to global

warming predictions: the effects of decadal climate oscillations. *J. Biogeogr.* **37,** 423-431.

Hochachka, P. W. and Somero, G. N. (2002). Biochemical Adaptation: Mechanism and Process in Physiological Evolution. Oxford: Oxford University Press. Pp 478.

Jiang, X. and Wang, X. (2004). Cytochrome c-mediated apoptosis. *Annu. Rev. Biochem.* **73**, 87-106.

Jones, S.J., Lima, F.P. and Wethey, D.S. (2010). Rising environmental temperatures and biogeography: poleward range contraction of the blue mussel *Mytilus edulis* L. in the western Atlantic. *J. Biogeogr*. **37**, 2243-2259.

Jones, S.J., Mieszkowska, N. and Wethey, D.S. (2009). Linking thermal tolerances and biogeography: *Mytilus edulis* (L.) at its southern limit on the east coast of the United States. *Biol. Bull*. **217**, 73-85.

Kefaloyianni, E., Gourgou, E., Ferle, V., Kotsakis, E., Gaitanaki, C. and Beis, I. (2005). Acute thermal stress and various heavy metals induce tissue-specific pro- or antiapoptotic events via the p38-MAPK signal transduction pathway in *Mytilus galloprovincialis* (Lam.), *J. Exp. Biol*. **208**, 4427-4436.

Klobučar, G. I.V., Štambuk. A., Hylland. K. and Pavlica. M. (2008). Detection of DNA damage in haemocytes of *Mytilus galloprovincialis* in the coastal ecosystems of Kaštela and Trogir bays, Croatia. *Sci. Total Environ.* **405**, 330-337.

Końca, K., Lankoff. A., Banasik. A., Lisowska, H., Kuszewski, T., Góźdź, S., Koza, Z. and Wojcik, A. (2003). A cross-platform public domain PC image-analysis program for the comet assay. *Mutat. Res.* **534**, 15-20.

Kültz, D. (2005). Molecular and evolutionary basis of the cellular stress response. *Annu. Rev. Physiol*. **67**, 225-257.

Lakhani, S.A., Masud, A., Kuida, K., Porter, G.A. Jr, Booth, C.J., Mehal, W.Z., Inayat, I. and Flavell, R.A. (2006). Caspases 3 and 7: key mediators of mitochondrial events of apoptosis. *Science* **311**, 847-51.

Li, H., Toubiana, M., Monfort, P. and Roch, P., (2009). Influence of temperature, salinity and *E. coli* tissue content on immune gene expression in mussels: Results from a 2005–2008 survey. *Dev. Comp. Immunol*. **33**, 974–979.

Li, J. J. and Deshaies, R. J. (1993). Exercising self-restraint: discouraging illicit acts of S and M in eukaryotes *Cell* **74**, 223–226.

Lockwood, B. L., Sanders, J. G. and Somero, G. N. (2010). Transcriptomic responses to heat stress in invasive and native blue mussels (genus *Mytilus*): molecular correlates of invasive success. *J. Exp. Biol*. **213**, 3548-3558.

Lockwood, B.L. and Somero, G.N. (2011). Invasive and native blue mussels (genus *Mytilus*j) on the California coast; the role of physiology in a biological invasion. *J. Exp. Mar. Biol. Ecol.* **400**, 167-174.

Lowe, D. M. and Pipe, R. K. (1994). Contaminant induced lysosomal membrane damage in marine mussel digestive cells: An *in vitro* study. *Aquat. Toxicol*. **30**, 357–365.

Lowe, D. M., Fossato, V. U. and Depledge, M. H. (1995). Contaminant induced lysosomal membrane damage in blood cells of mussels *M. galloprovincialis* from the Venice Lagoon: An in vitro study. *Mar. Ecol. Prog. Ser*. 129, 189–196.

Micic, M., Bihari, N., Jaksic, Z., Muller, W. E.G. and Batel, R. (2002). DNA damage and apoptosis in the mussel *Mytilus galloprovincialis*. *Mar. Environ. Res*. **53**, 243–262.

Murai, H., Hiragami, F., Kawamura, K., Motoda, H., Koike, Y., Inoue, S., Kumagishi, K., Ohtsuka, A. and Kano, Y. (2010). Differential response of heat-shock-induced p38 MAPK and JNK activity in PC12 mutant and PC12 parental cells for differentiation and apoptosis. *Acta. Med. Okayama* **64**, 55-62.

Nitiss, J. L. (1998). Investigating the biological functions of DNA topoisomerases in eukaryotic cells. Biochim. *Biophys. Acta* **1400:** 63-68.

Ori, Y., Herman, M., Weinstein, T., Chagnac, A., Zevin, D., Milo, G., Gafter, U. and Malachi, T. (2005). Spontaneous DNA repair in human peripheral blood mononuclear cells. *Biochem. Biophys. Res. Commun*. **320**, 578–586.

Parolini, M., Quinn, B., Binelli, A. and Provini, A. (2011). Cytotoxicity assessment of four pharmaceutical compounds on the zebra mussel (*Dreissena polymorpha*) haemocytes, gill and digestive gland primary cell cultures. *Chemosphere*. **84**, 91-100.

Prasad, R., Beard, W. A., Batra, V. K., Liu, Y., Shock, D. D. and Wilson, S. H. (2011). A eview of recent experiments on step-to-step "hand-off" of the DNA intermediates in mammalian base

763 excision repair pathways. *Mol. Biol. (Mosk).* **45,** 586–600.

764 **Roberts, K.P., Sobrino, J.A., Payton, J., Mason, L.B. and Turesky, R.J.** (2006). Determination
765 of apurinic/apyrimidinic lesions in DNA with high-performance liquid chromatography and
766 tandem mass spectrometry. *Chem. Res. Toxicol*. **19**, 300–309.

767 **Schneider, K.R.** (2008). Heat stress in the intertidal: comparing survival and growth of an
768 invasive and native mussel under a variety of thermal conditions. *Biol Bull*. **215**, 253-64.

769 **Singh, N.P., McCoy, M.T., Tice, R. and Schneider, E.L.** (1988). A simple technique for the
770 quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.* **175**, 184-191.

771 SPSS® Base 13.0 User'S Guide, (2004). SPSS Inc. 409-424.

772 **Tomanek, L. and Zuzow, M.J.** (2010). The proteomic response of the mussel congeners *Mytilus*
773 *galloprovincialis* and *M. trossulus* to acute heat stress: implications for thermal tolerance limits
774 and metabolic costs of thermal stress. *J. Exp. Biol.* **213**, 3559-3574.

775 **Wallace, S. S.** (1994). DNA damages processed by base excision repair—biological consequences.
776 Int. *J. Radiat. Biol*. **66**, 579–589.

777 **Wepener, V., Bervoets, L., Mubiana, V. and Blust, R.** (2008). Metal exposure and biological
778 responses in resident and transplanted blue mussels (*Mytilus edulis*) from the Scheldt estuary.
779 *Mar. Pollut. Bull.* **57**, 624 – 631.

780 **Yao, C.L., Wu, C.G., Xiang, J.H., Li, F.H., Wang, Z.Y. and Han, X.Z.** (2008). The lysosome
781 and lysozyme response in Chinese shrimp *Fenneropenaeus chinensis* to *Vibrio anguillarum* and
782 laminarin stimulation. *J. Exp. Mar. Biol. Ecol.* **363**, 124-129.

Figure legends.

Fig. 1. Measurements of DNA damage in hemocytes of mussels following temperature stress. Cell: a mussel hemocyte after single-cell gel electrophoresis; Head: head diameter; Tail: tail length; Stain: SYBR green.

Fig. 2. Single-stranded DNA breakage in hemocytes of mussels after acute cold- and heat stress. A. *M. californianus*, B. *M. galloprovincialis* (13ºC = control). Statistical significance was determined by two-way ANOVA with temperature and stress time as factors. Asterisks denote statistically significant differences ($p<0.05$) between experimental treatments (high- and low temperature stress for varying times) and the control (13°C specimens).

Fig. 3. Double-stranded DNA breakage in hemocytes of mussels after heat and cold stress (13ºC = control). A. *M. californianus* B. *M. galloprovincialis*. Statistical significance was determined by two-way ANOVA with temperature and stress time as factors. Asterisks denote statistically significant differences ($p<0.05$) between experimental treatments (high- and low temperature stress for varying times) and the control (13°C specimens).

Fig. 4. Analysis of phosphorylation on p38 (Thr180/Tyr182) and phosphorylation on JNK/SAPK (Thr-183/ Tyr-185) in hemocytes of *M. californianus* and *M. galloprovincialis* after low- and high-temperature stress (13ºC = control). A. phospho-p38 (Thr-180/Tyr-182) in *M. californianus*; B. phospho-p38 (Thr-180/Tyr-182) in *M. galloprovincialis*; C. phospho-JNK1/SAPK (Thr-183/ Tyr-185, p46 band) in *M. californianus*; D. phospho-JNK1/SAPK (Thr-183/ Tyr-185, p46 band) in *M. galloprovincialis*; E. phospho-JNK2/3 (Thr-183/ Tyr-185, p46 band) in *M. californianus*; F. phospho-JNK2/3 (Thr-183/ Tyr-185, p46 band) in *M. galloprovincialis*; Statistical significance was determined by two-way ANOVA with temperature and stress time as factors. Asterisks denote statistically significant differences ($p<0.05$) between experimental treatments (high- and low temperature stress for varying times) and the control (13°C specimens).

Fig. 5. Analysis of pro- and active caspase-3 expression in hemocytes of *M. californianus* and *M. galloprovincialis* after acute cold- and heat stress. A. expression of pro-caspase-3 in *M. californianus*; B. expression of pro-caspase-3 in *M. galloprovincialis*; C. expression of active caspase-3 in *M. californianus*; D. expression of active caspase-3 in *M. galloprovincialis*. Statistical significance was determined by two-way ANOVA with temperature and stress time as factors. Asterisks denote statistically significant differences ($p<0.05$) between experimental treatments (high- and low temperature stress for varying times) and the control (13°C specimens).

Fig. 6. Numbers of non-viable hemocytes after acute cold- and heat stress. *M. californianus* (A) and *M. galloprovincialis* (B). Statistical significance was determined by two-way ANOVA with temperature and stress time as factors. Asterisks denote statistically significant differences ($p<0.05$) between experimental treatments (high- and low temperature stress for varying times) and the control (13°C specimens).

Fig. 7. Mean NRR times in hemocyte lysosomes of *M. californianus* (A) and *M. galloprovincialis* (B) at different time intervals of exposure to acute cold- and heat stress. Statistical significance was determined by two-way ANOVA with temperature and stress time as factors. Asterisks denote statistically significant differences ($p<0.05$) between experimental treatments (high- and low temperature stress for varying times) and the control (13°C specimens).

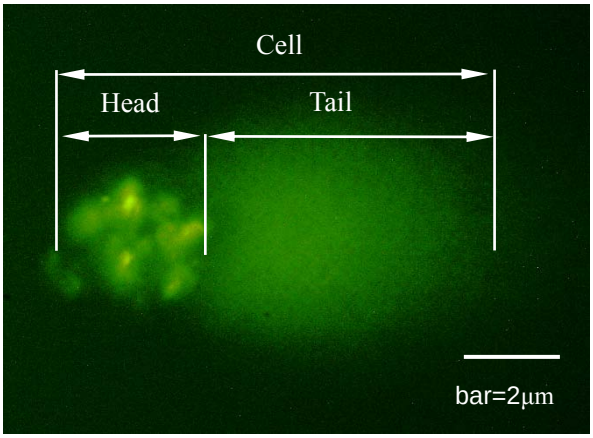
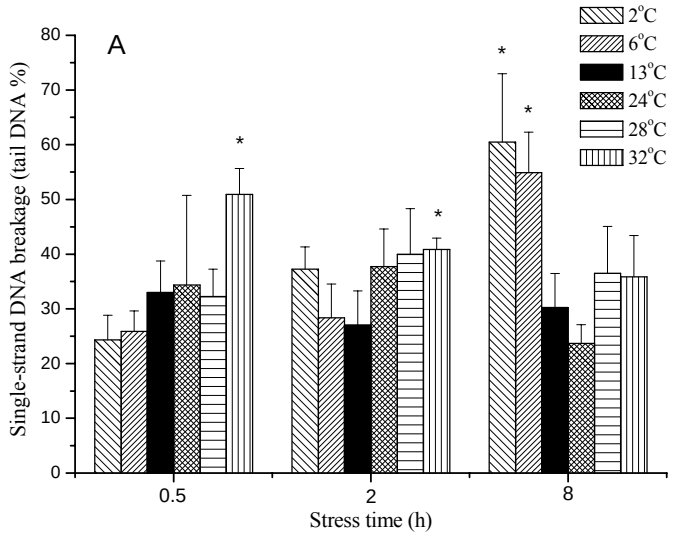


Fig. 1.



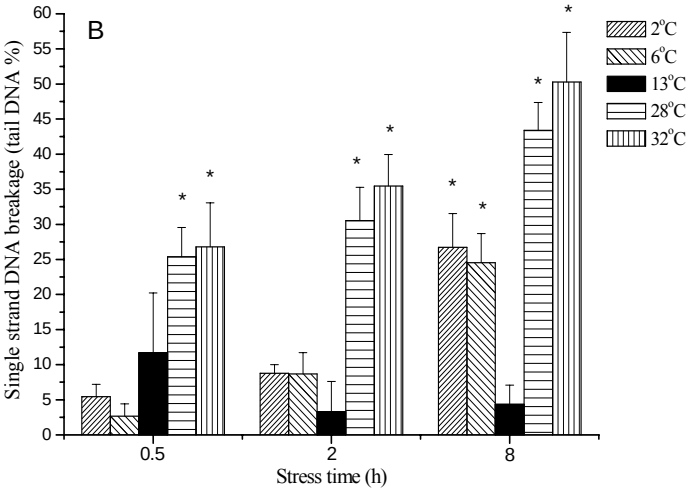
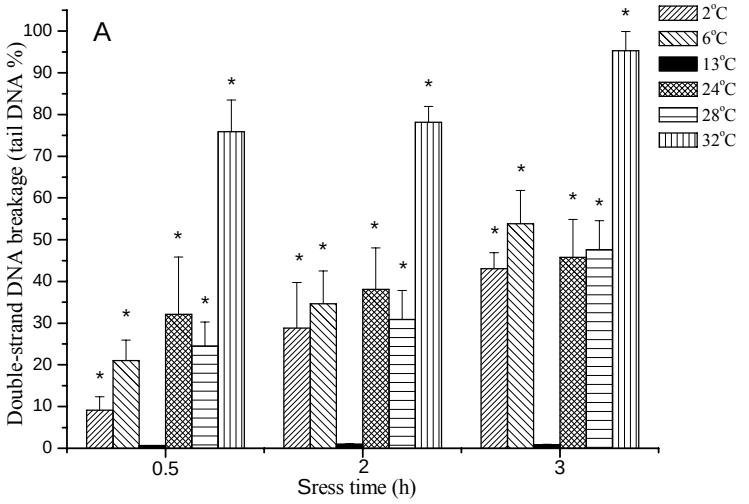


Fig. 2.



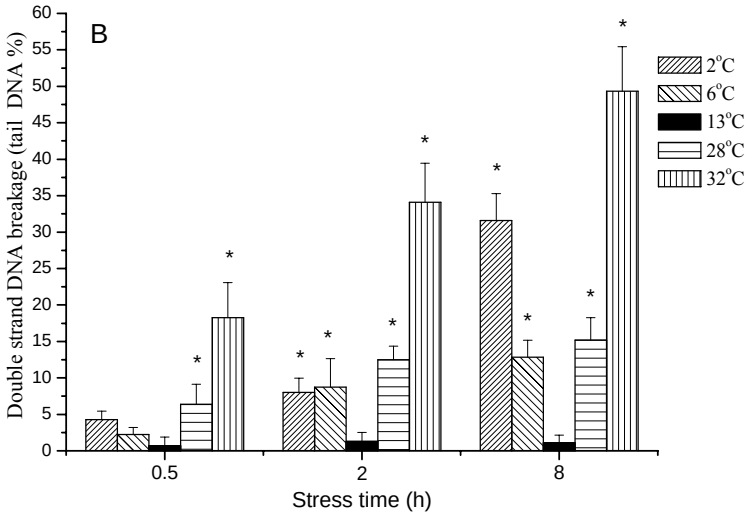
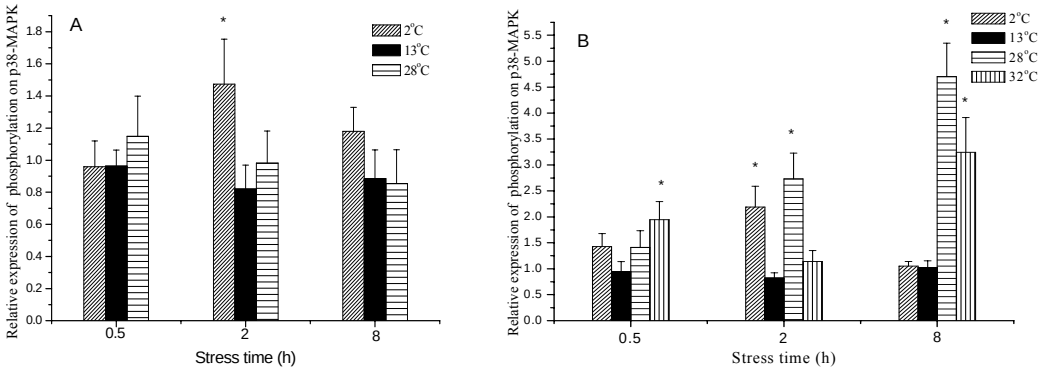
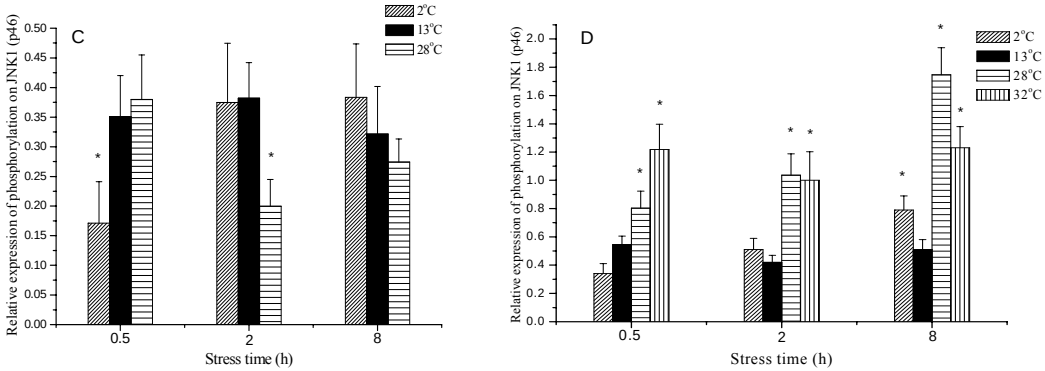


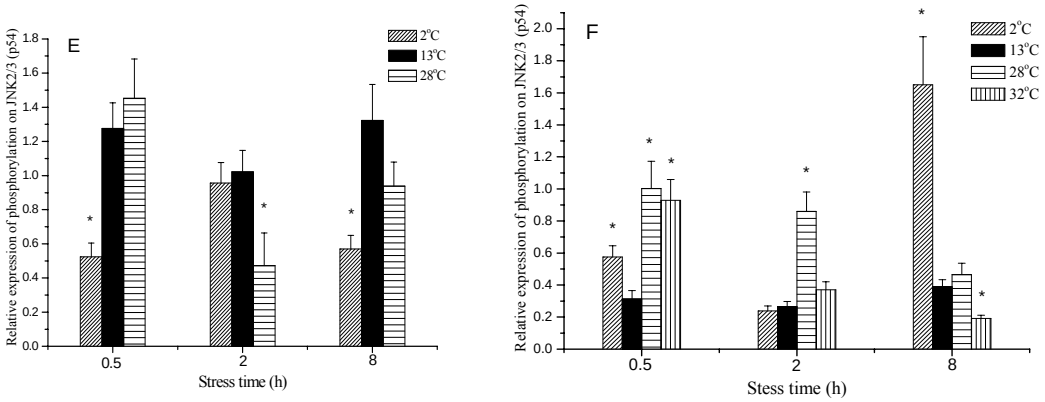
Fig. 3.



912



913



914

915

916

917 Fig. 4.

918

919

920

921

922

923

924

925

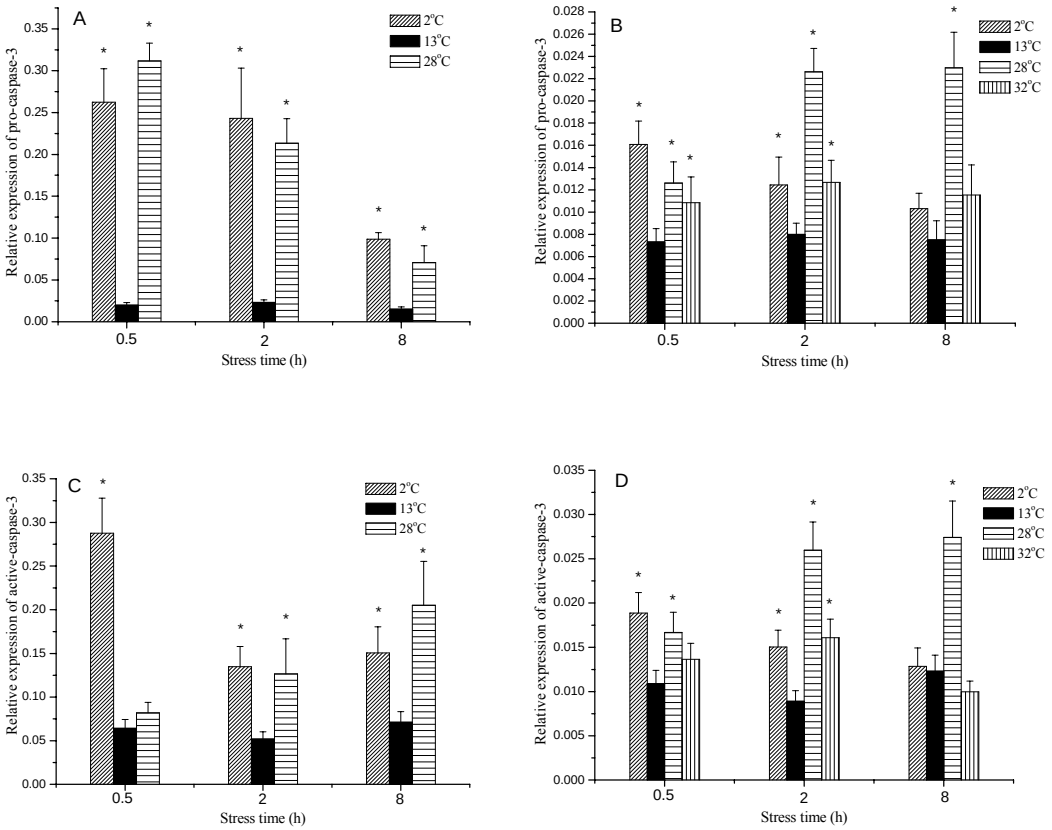
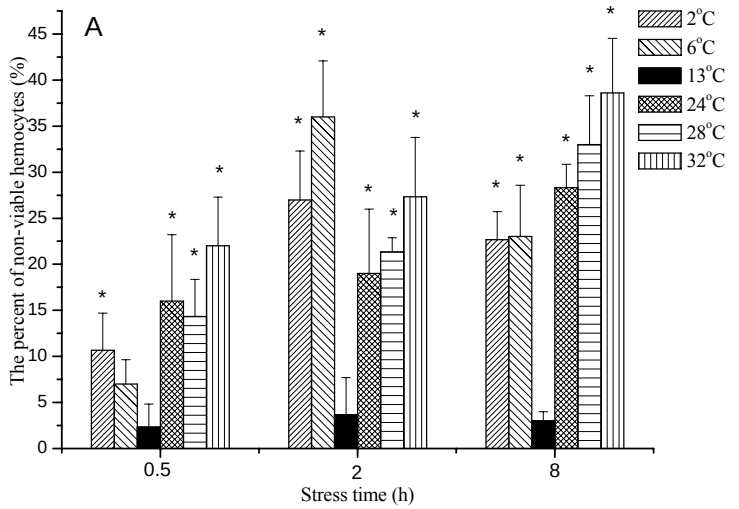


Fig. 5.



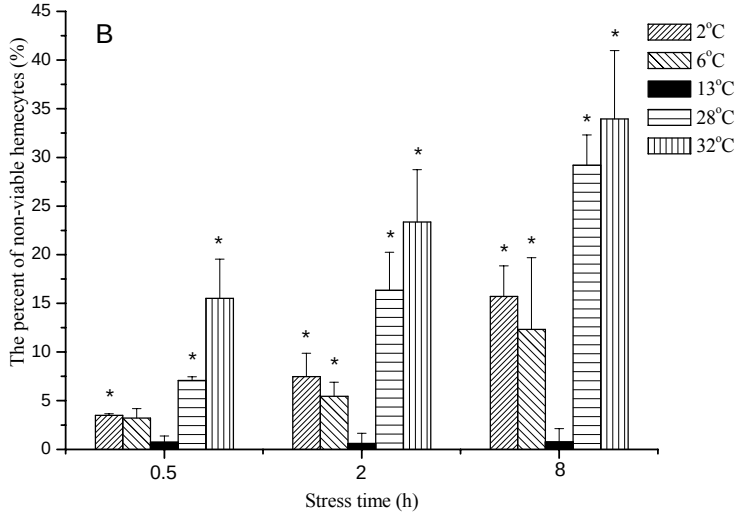
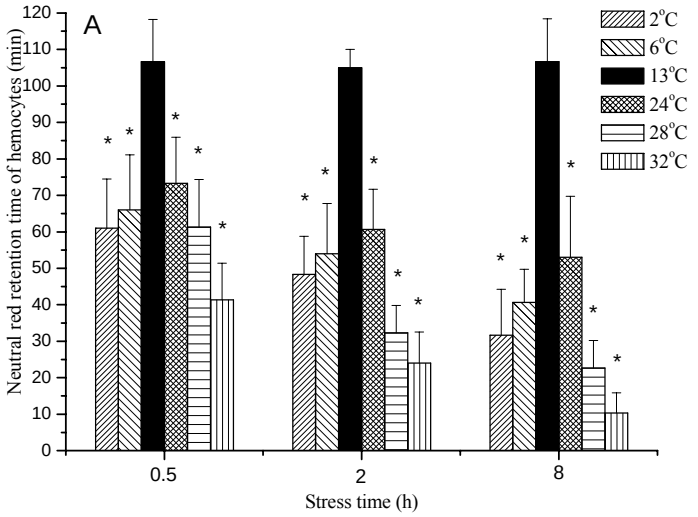


Fig. 6.



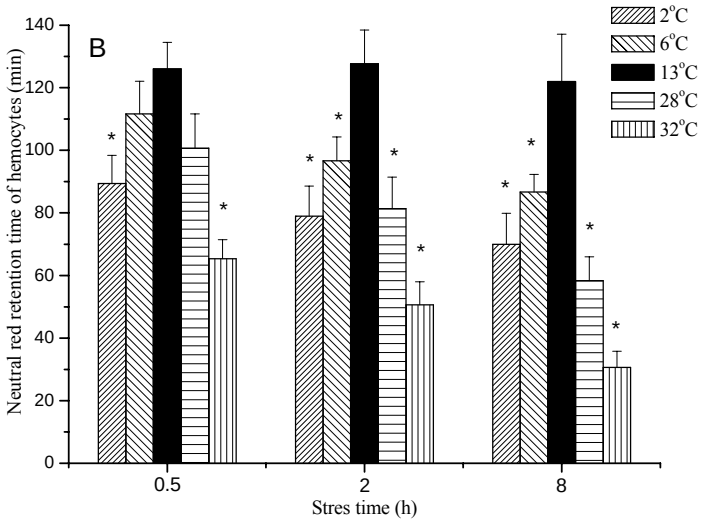


Fig. 7.

982