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RESEARCH REPORT

Heat stress induces ROS production and histone phosphorylation in celomocytes of *Eisenia hortensis*

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Abstract

The effect of heat stress on celomocytes (leukocytes) from *Eisenia hortensis* was investigated by measuring the production of reactive oxygen species (ROS). After culturing celomocytes at temperatures ranging from 4 °C (control) to 44 °C for 3-16 h, ROS levels were measured using a flow cytometric method employing dihydrorhodamine 123 (DHR123) for ROS detection and 7-aminoactinomycin D (7-AAD) as a viability stain. Reproducibly we observed significant ($p < 0.05$) increases in ROS production and decreases in cell viability at temperatures of 28 °C and above. We then examined the effect of heat stress on histone phosphorylation employing antibodies specific for γH2AX as an indicator of histone modification. Celomocytes were incubated at temperatures ranging between 20 °C to 35 °C for 16 h and antibodies specific for phosphorylated serines in H2AX histones were employed through flow cytometric analysis. Comparing controls to heat-stressed samples using three separate assays reproducibly confirmed significant H2AX phosphorylation ($p < 0.05$). Collectively, these results emphasize the importance of selecting appropriate temperatures for rearing invertebrates in laboratory-based habitats and for culturing invertebrate cells when conducting *in vitro* assays in order to minimize oxidative stress. The possible cellular effects of heat stress in soil ecosystems associated with global warming events is also considered.

Key Words: reactive oxygen species (ROS); γH2AX; histone phosphorylation; heat stress; *in vitro* cell culture

Introduction

Reactive oxygen species (ROS) are highly reactive molecules used during innate host defense mechanisms to eradicate pathogens and initiate programmed cell death signal cascades (Bolwell, 1996; Forman and Torres, 2002; Pastori, 2002; Reth, 2002). ROS are produced by phagocytes such as neutrophils and macrophages and are a universal indicator of oxidative cellular stress (Finkel, 2001; Fuller-Espie *et al.*, 2010). The formation of ROS from ground state oxygen takes place through a series of reductions provided by either energy transfers or by electron transfer reactions. This series of reactions leads to the formation of superoxide radical ions, which are reduced to hydrogen peroxide and then reduced to a hydroxyl radical through a series of electron transfers (Klotz, 2002; Apel and Hirt, 2004). ROS is a natural byproduct of metabolism, but when an excess of these highly radical molecules accumulate during times of stress, the ability of the cell to neutralize intermediates or repair their toxic effects is overwhelmed leading to damage of cellular membranes, DNA, neighboring cells and tissues through the induction of inflammatory events (Duval *et al.*, 2003; Fuller-Espie *et al.*,2010).

ROS resulting in DNA damage have been shown to be produced through a variety of environmental stimuli including ionizing radiation, chemical mutagens, as well as heat (Beckman and Ames, 1998; Bruskov *et al.*, 2002). The effect of heat stress on organisms has been studied and confirmed to increase the production of ROS, possibly by interfering with electron transport assemblies of the inner mitochondrial membrane and may be a factor resulting in disruption of biological molecules such as DNA, proteins, and lipids (Ando *et al.*, 1997; Bruskov *et al.*, 2002; Mujahid *et al.*, 2005). Invertebrate heat-induced ROS is a concern for laboratories frequently using invertebrate experimental organisms for *in vitro* assays. In addition, organisms not reared at appropriate temperatures in laboratory-based habitats may generate ROS leading to perturbation

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of results observed in both *in vivo* and *in vitro* studies. In our lab, various *in vitro* cellular assays are conducted routinely including innate immune response assays to investigate pathogen-associated molecular patterns (PAMPs) and xenobiotics, natural killer cell-like activity assays, and phagocytosis assays (Patel *et al.*, 2007; Fuller-Espie, 2010; Nacarelli and Fuller-Espie, 2011; Fuller-Espie *et al.*, 2011).

The primary goal of this *in vitro* study was to investigate the effect of heat stress on the celomocytes of *Eisenia hortensis* (also known as the European nightcrawler) and to determine the temperature threshold which triggers increased generation of ROS for optimal *in vitro* studies. To determine the onset of heat-induced cellular damage, ROS production was detected through a range of temperatures along with analyzing cellular viability and H2AX histone phosphorylation. The generation of DNA damage was observed through double-stranded breaks which were identified by phosphorylated histone protein H2AX at the flank of the double-strand break at Ser-139 at the C-terminus (Shimohara *et al.*,2008). Phosphorylation is mediated by DNA-dependent protein kinases and the resulting phosphorylated form of H2AX is referred to as gamma-H2AX (γH2AX) (Rogakou *et al.*, 1999). H2AX is unique and is highly conserved between eukaryotes (Madigan *et al.*, 2002). Furthermore, it has been reported that antibodies that specifically bind phosphorylated H2AX in mammals also recognize radiation-induced proteins in *Muntiacus muntjac*, *Xenopus laevis* and *Drosophila melanogaster* (Rogakou *et al.*, 1999). This observed conservation between various organisms made the H2AX protein an ideal target for working with *E. hortensis*.

Earthworm celomocytes can be studied *in vitro* through experimental extrusion through the dorsal pores of the celomic cavity where the celomocytes are located. Three distinct subpopulations of celomocytes exist which are believed to derive from a single progenitor cell (prohemocyte): phagocytic hyaline amebocytes (large celomocytes), granular natural killer-like amebocytes (small celomocytes), and eleocytes which contain chloragosomes which are used to secrete lytic substances (Engelmann *et al.*, 2004; Hartenstein, 2006; Fuller-Espie *et al.*, 2008). Post extrusion, cells are analyzed with flow cytometry allowing simultaneous measurements of multiple characteristics of a single cell at a rapid rate. Flow cytometry measures relative size (forward scatter), the granularity or internal complexity (side scatter) (allowing the differentiation between subpopulations), and also fluorescence associated with cellular subpopulations. In our experiments relative fluorescence intensity using fluorochromes was measured by the instrument to determine: 1) cellular viability using 7-aminoactinomycin D (7-AAD) which only binds to double-stranded nucleic acid and fluoresces upon entry into a non-viable, membrane-compromised cell; 2) ROS production upon oxidation of the reporter molecule dihydrorhodamine 123 (DHR 123); and 3) histone phosphorylation using antibodies specific for γH2AX and fluorescein isothiocyanate(FITC)-conjugated secondary antibodies (Rabinovitch *et al.*, 1986; Nicoletti *et al.*, 1991; Huang and Darzynkiewicz, 2006).

Materials and Methods

Reagents

General laboratory reagents and plastic ware were purchased from Fisher Scientific unless otherwise noted.

Cell culture

All cell culture reagents and phosphate-buffered saline (PBS) were purchased from Invitrogen unless otherwise noted. Dulbecco's Modified Eagle Medium (DMEM) was supplemented with 10% Serum Supreme (Lonza Bio Whittaker), 100 µg/ml ampicillin (Shelton Scientific), 10 µg/ml kanamycin (Shelton Scientific), 10 µg/ml tetracycline, 5 µg/ml chloramphenicol (Fluka Biochemika), 1X penicillin/streptomycin/amphotericin B (Gibco), 1X nonessential amino acids (Gibco) and 1X L-glutamine (Gibco) to comprise Super DMEM (SDMEM). All assays were conducted *in vitro* post celomotycte extrusion. The 3 h incubation experiments were conducted in water baths in test tubes. The 16 h incubations were conducted in 5% CO_2 incubators in 96-well V-bottom plates.

Earthworm husbandry

E. hortensis were purchased from Vermitechnology Unlimited, Orange Lake, Florida, USA, who import *E. hortensis* from Star Food, Holland, Scherpenzeelseweg 95, 3772ME Barneveld, The Netherlands. Species identity was determined by the United States Department of Agriculture, USDA Permit #52262 (Vermitechnology, personal communication). Short-term colonies were maintained at room temperature (RT) in the dark on autoclaved pine wood chips and shredded paper moistened with water and Single Grain Rice Cereal or Rice with Bananas Cereal (Gerber) until use. Bedding and food were changed twice weekly.

Extrusion of celomocytes

Prior to experimentation, earthworms were chosen based on their color and activity; earthworms with healthy deep coloration, lacking yellow appearance, and with high activity were placed overnight on moist paper towels saturated with 2.5 µg/ml Fungizone (Fisher Scientific) to reduce the level of fecal material and other surface contaminants. To collect celomocytes from an earthworm, earthworms were extruded according to Bearoff and Fuller-Espie (2011). Briefly, earthworms were placed in a plastic autoclaved trough containing 3 ml of FACS Flow sheath fluid (BD Biosciences) used as an extrusion buffer. The celomocytes were then transferred to 0.5 ml Accumax (Innovative Cell Technology) for a 5 min incubation period at RT. Then the cells were washed with 5 ml PBS prior to centrifugation at 150 Xg for 5 min at 4 °C. Celomocytes were resuspended in 1 ml or 0.5 ml SDMEM and enumerated using a hemocytometer. Only large celomocytes (LC) and small celomocytes (SC) were included in the cell count; eleocytes (which are highly autofluorescent) were not counted but did

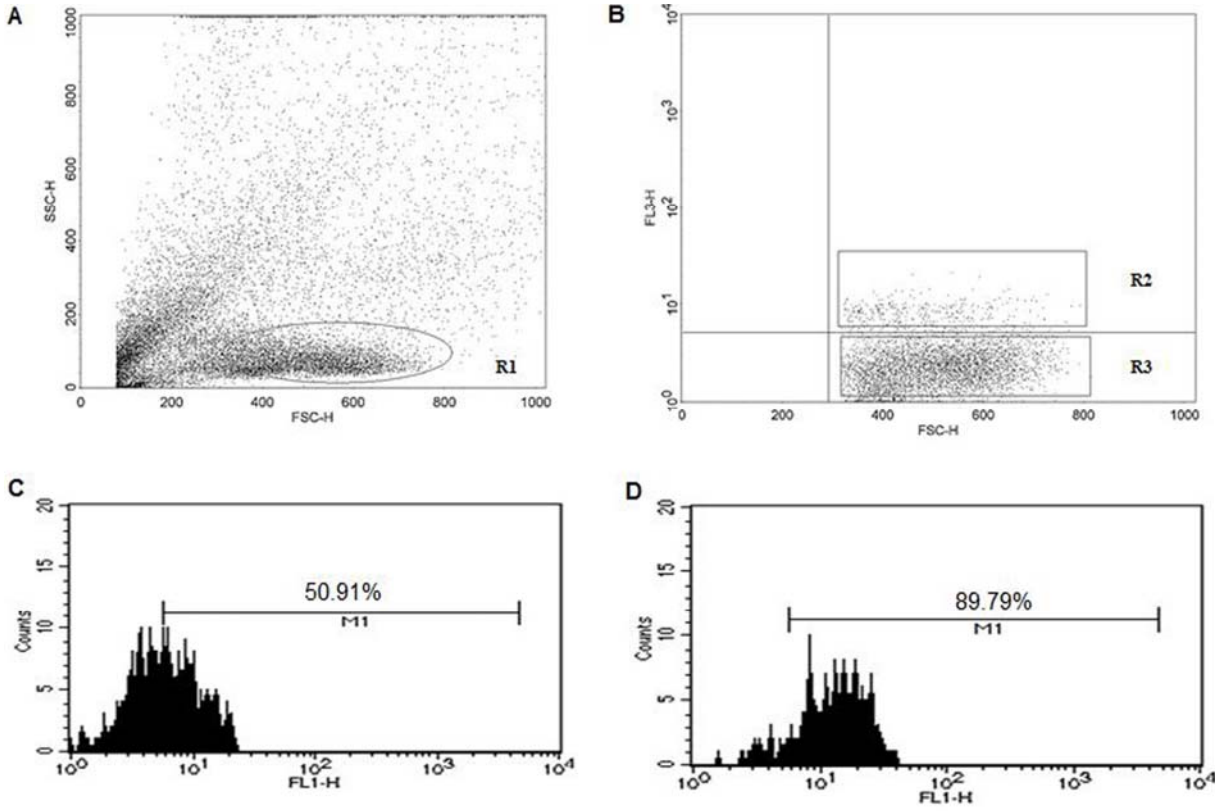

Fig. 1 Representative flow cytometry profiles for ROS and viability assays. (A) depicts a typical celomocyte profile produced post flow cytometric acquisition of FSC (size) (abscissa) versus SSC (granularity) (ordinate) of celomocytes where R1 = granular amebocytes, which were the desired celomocyte subpopulation for this study. (B) shows FSC (abscissa) versus FL-3 fluorescence (ordinate) of R1-gated celomocytes to distinguish viable from non-viable cells. R2 depicts non-viable 7-AAD positive cells (FL-3 positive) and R3 depicts viable 7-AAD negative cells (FL-3 negative). (C and D) show FL-1 (abscissa) versus cell number (ordinate) of amebocytes gated on both R1 and R3. Control (C) and heat-treated (D) samples are shown comparing temperatures 20 °C to 35 °C, respectively. FSC = forward scatter; SSC = side scatter; FL-1 = relative fluorescence intensity of DHR123; FL-3 = relative fluorescence intensity of 7-AAD.

factor into a quality score. Samples with large numbers of eleocytes compared to LC and SC were not used in assays.

For preliminary ROS production and cellular viability assays, individual earthworms (n = 3) were selected and celomocytes were extruded and analyzed in duplicate. During later ROS production and cellular viability assays, as well as γH2AX assays, selected celomocytes from approximately seven earthworms were batched and assays were performed in duplicate or triplicate as indicated.

Detection of cell viability and ROS

The viability dye 7-aminoactinomycin D (7-AAD; BD Biosciences) was employed for two purposes: 1) to enable the elimination of dead cells from our ROS analyses; and 2) to determine percent viable cells. For positive controls, celomocytes (10^5 cells in 0.2 ml) were pretreated with saponin (0.1%) at 25 °C. 7-AAD fluorescence was measured using the FL-3 detector. Cell viability was measured after establishing a threshold to distinguish live versus dead cells using saponin-treated celomocytes as the dead cell indicator (see Fig. 1). The higher the percent of cells entering the FL-3 positive region (R2) of the saponin control, the lower the percent cell viability.

ROS production was measured in viable celomocytes by gating on small celomocytes that were 7-AAD negative using dihydrorhodamine 123 (DHR 123; Invitrogen, 1 μM in SDMEM) which was converted to rhodamine 123 upon oxidation. For positive controls, celomocytes (10^5 cells in 0.2 ml) were pretreated with H_2O_2 (67.6 mM), or $CdCl_2$ (500 μM) (data not shown). Rhodamine 123 fluorescence was measured using the FL-1 detector.

Fixation of celomocytes for γH2AX analysis

Celomocytes from 7 earthworms were pooled (batched) and used at 2-2.5 x 10^5 in 0.2 ml and incubated at temperatures ranging from 20 °C – 35 °C in three separate assays in duplicate or triplicate. Following heat treatment, celomocytes were fixed according to Huang and Darzynkiewicz (2006) with

minor modification. Celomocytes were centrifuged (150 Xg, 5 min, 4 °C) and treated with 0.2 ml ice-cold 1% methanol-free formaldehyde. Following a 15 min incubation celomocytes were centrifuged and then resuspended in ice-cold 70% ethanol. After fixation (up to one week at -20 °C) the celomocytes were centrifuged, the ethanol was removed, and cells were washed twice in 200 µl 1% bovine serum albumin, 0.2% v/v Triton X-100 in PBS (BSA-T-PBS) before adding blocking serum.

Immunofluorescence - γH2AX antibodies

Detection of γH2AX using flow cytometric analysis was performed according to Huang and Darzynkiewicz (2006) with minor modification. Post-fixation and prior to primary antibody incubations, cells were incubated in 10% blocking serum (mouse serum sc-45051) for 20 min at RT. γH2AX was detected using primary rabbit polyclonal anti-γH2AX antibody specific for the phosphorylated serine 129 (sc-101696). In addition isotype-matched control normal rabbit IgG (sc-3888) antibody was used to ensure antibody specificity. Secondary mouse anti-rabbit IgG FITC-conjugated antibody (sc-2359) was used. All antibodies and sera were ordered from Santa Cruz Biotechnology, Inc. and used at the concentrations recommended by the supplier.

Flow cytometric analyses

Flow cytometry was carried out using a FACSCalibur flow cytometer (BD Biosciences). LISTMODE data was acquired and analyzed using Cell Quest Pro software. Fig. 1 illustrates the approach used to analyze ROS production. First, a two-parameter dot plot depicting forward scatter (FSC) versus side scatter (SSC) was created (Fig. 1A). Region 1 (R1) was placed around the granular amebocytes. Next, a two parameter dot plot gated on the R1 subpopulation measured forward scatter versus 7-AAD relative fluorescence intensity (FL-3), and two additional regions were placed on the cellular events. R2 represents the 7-AAD-positive population (non-viable cells), and R3 represents the 7-AAD-negative population (viable cells) (Fig. 1B).

After exposure to heat, ROS production in live granular amebocytes was measured using the fluorogenic substrate DHR 123, a probe widely used to measure intracellular H_20_2. In order to detect ROS production a single parameter histogram gated on both R1 and R3 population measured rhodamine 123 (FL-1). Therefore, the final analysis was restricted to live granular amebocytes. Fig. 1 shows representative histograms comparing a 20 °C control (Fig. 1C) versus a 35 °C heat-stressed sample (Fig. 1D). Marker 1 (M1) was set at approximately the median (~50%) of the control histogram as a baseline for comparison. Events were considered positive if they exhibited a higher relative fluorescence intensity than the median fluorescence value obtained in the control cells. Figure 1C shows the 20 °C control sample with a value of 50.91% compared to the 35 °C heat-stressed sample shown in Figure 1D which has a value of 89.79%. Preliminary samples were run in duplicate (Figs 2A and 2B) whereas later assays were run in triplicate (Figs 2C and 2D) and average values were graphed showing standard deviation.

Statistical Analysis

All graphs and data analysis were created and processed using Microsoft Excel 2007. Student's t-test paired two samples for means was used with a 95% confidence interval to determine statistical significance.

Results

Flow cytometric detection of ROS production and viability

Healthy celomocytes were identified through the use of the viability dye 7-AAD which enters only membrane-compromised cells and upon binding to double-stranded nucleic acid fluoresces, thereby indicating non-viability. Therefore, 7-AAD negative cells exhibiting no fluorescence could be preferentially selected for final analysis of rhodamine 123 (FL-1 detector) by creating regions and gating on desired subpopulations. Cells undergoing late apoptotic or necrotic pathways are considered non-viable (Rabinovitch *et al.*, 1986; Bearoff and Fuller-Espie, 2011). In order to identify viable cells a two parameter dot plot gated on the R1 subpopulation measured forward scatter versus 7-AAD relative fluorescence intensity (FL-3) and consisted of two additional regions (R2 and R3) which were utilized to determine the effects of heat stress on cellular viability (Fig. 1B).

As a starting point, a wide range of incubation temperatures (4 °C - 44 °C) was employed in duplicate to examine ROS production and viability. Figure 2A illustrates the ROS results while Figure 2B shows the viability results for this temperature range. In this experiment, the 4 °C sample was the control to which the others were compared, and a 3 h incubation period was used.

This lead to further experiments that expanded the temperature range to represent their natural habitat more closely (20 °C – 35 °C) and also increased the duration of incubation to 16 h. Three additional assays were performed using either individual earthworms in duplicate or triplicate (data not shown), or batches of earthworms (7 per batch) in triplicate. Figure 2C shows ROS production and Figure 2D shows viability, both representative of data acquired for all three assays. Here the 20 °C sample was the control to which the others were compared. Statistically significant ($p \leq 0.05$) results were obtained for viability assays at temperatures of 25 °C and higher, and for ROS assays at temperatures of 30 °C and higher for the majority of batches examined. Therefore, these results clearly show increased cell death and ROS production as temperatures increase above 25 °C or 30 °C, respectively.

Flow cytometric detection of H2AX phosphorylation

Figure 3A illustrates the specificity of the anti-γH2AX antibody that was used in this study and the degree of nonspecific binding of antibodies to celomocytes. Celomocyte samples incubated at 35°C and subsequently fixed were stained with either an isotype-matched control antibody (left curve) or the anti-γH2AX antibody (right curve). This result shows that the anti-γH2AX antibody binds to celomocytes with specificity. Figure 3B depicts a

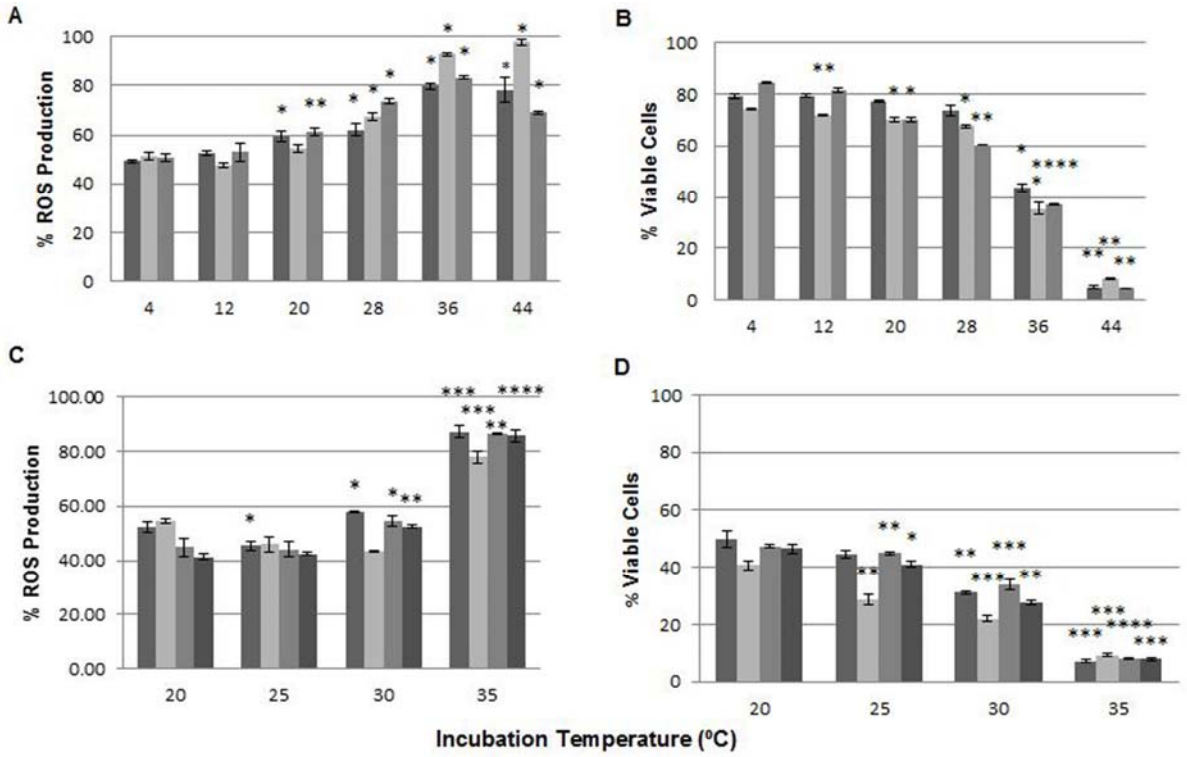

Fig. 2 Heat-stressed granular amebocytes exhibit increased ROS production and decreased cell viability. (A and C) are gated on both R1 and R3 (see Fig. 1) and incubation temperature (abscissa) versus the percent increase in ROS production above the median for the untreated control (ordinate) (A control = 4 °C; C control = 20 °C) are shown. (B and D) indicate the percent of all cells in R1 that are also included in R3 (see Fig. 1) showing incubation temperature (abscissa) versus the percent of viable cells post-treatment (ordinate). Each bar color corresponds to an individual earthworm analyzed in duplicate (A and B) or the average of different batches of earthworms (n=7) analyzed in triplicate (C and D). * = $p \leq 0.05$; ** = $p \leq 0.005$; *** = $p \leq 0.0005$; **** = $p \leq 0.00005$. Error bars signify standard deviation.

histogram overlay comparing a control sample (25 °C) to a heat-treated sample (35 °C) measuring H2AX phosphorylation. It shows the positioning of the M1 marker set at the approximate median of the control sample. Figures 3C and 3D are representative of results obtained from a total of three assays. The assay depicted in Figure 3C was conducted in duplicate at incubation temperatures of 20 °C, 25 °C, 30 °C, or 35 °C, and the control was the 20 °C sample. The assay depicted in Figure 3D was conducted in triplicate at incubation temperatures of 25 °C, 30 °C, 32.5 °C, or 35 °C and the control was the 25 °C sample. These results indicate that statistically significant ($p \leq 0.05$) heat-induced H2AX phosphorylation is taking place in celomocytes at 30 °C and above compared to controls.

Discussion

This study demonstrates that heat stress (28 °C and above) causes ROS production, decreases cell viability, and results in the phosphorylation of the H2AX histone in *E. hortensis* and therefore appropriate temperatures for rearing organisms and conducting cell culture assays must be established and implemented. Avoiding heat-induced stress will serve to minimize undesired background when conducting in vitro cellular assays. Although the mechanism of interaction between ROS and γH2AX is not well understood, it has been suggested that ROS production may be induced by the accumulation of γH2AX during DNA damage response (DDR) which results in γH2AX regulation of NOX1-mediated ROS generation post-DNA damage (Kang *et al.*, 2012). γH2AX has been called "the histone guardian of the genome" and seems to play an intricate role in DDR as the "marker of DNA breaks" signaling the need for DNA repair survival or apoptosis (Fernadez-Capetillo *et al.*, 2004; Cook *et al.*, 2009). Although a homolog of p53 has not been identified in *E. hortensis*, it is still of interest to note that a relationship between γH2AX-induced ROS generation seems to play a role in upstream signals that trigger p53 activation (Bragado *et al.*, 2007; Liu *et al.*, 2008). Although this study focused on heat stress in the earthworm, it is interesting to note that recently the effect of gene expression in earthworms in response to cold stress conditions was investigated leading to the construction of a cold-response regulatory-gene network (Kim *et al.*, 2012).

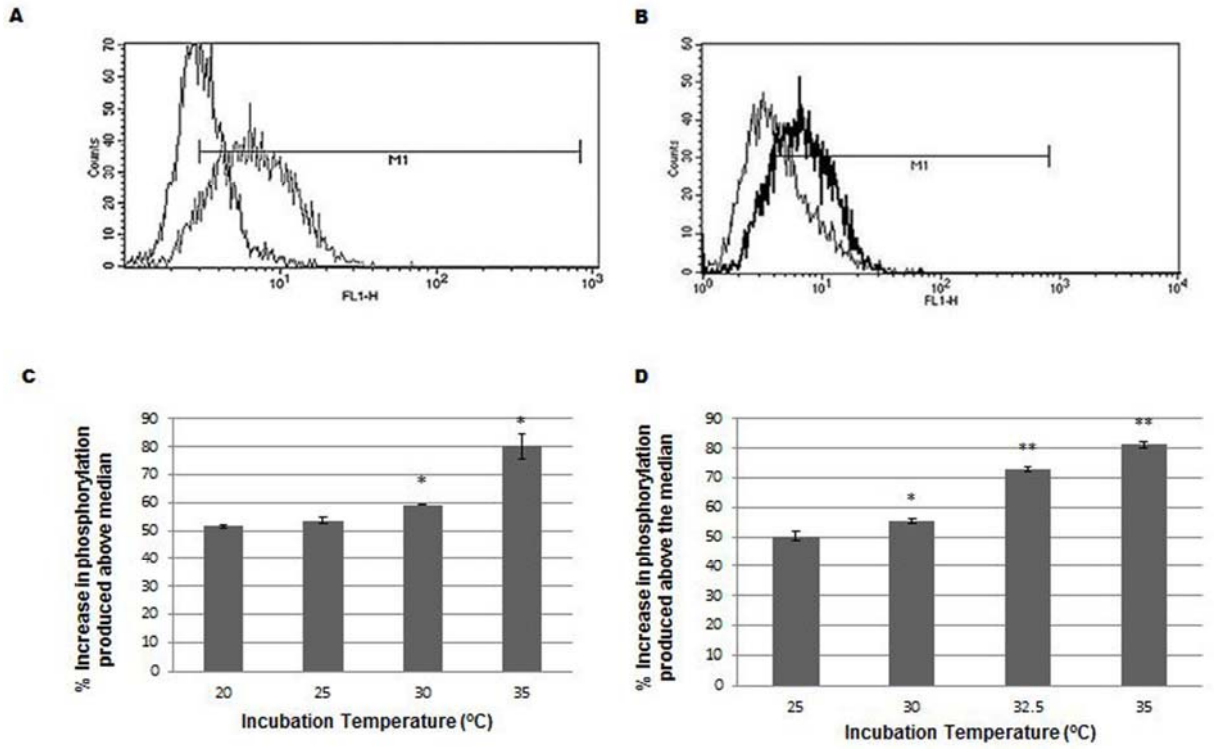

Fig. 3 Heat stress induces phosphorylation of H2AX. (A) shows representative FL-1 (abscissa) versus cell number (ordinate) on a histogram overlay between a control sample incubated with isotype-matched antibody (left curve) and a sample incubated with anti-γH2AX antibody (right curve), both heat-treated at 35 °C utilizing the M1 marker method to determine antibody specificity as previously stated. (B) shows representative FL-1 (abscissa) versus cell number (ordinate) on a histogram overlay between a control sample (25 °C) (not bolded) and a heat-treated sample (35 °C) (bolded), both incubated with anti-γH2AX antibody, and indicates the placement of the M1 marker method stated previously. (C) and (D) show incubation temperature (abscissa) versus the percent increase in H2AX phosphorylation (ordinate) above the median at which the M1 marker is set for the untreated control at 20 °C (C) or 25 °C for (D) in two separate assays. FL-1 = relative fluorescence intensity of FITC. Each bar represents a batch of earthworms (n=7). * = $p \leq 0.05$, ** = $p \leq 0.005$.

ROS-induced DNA damage causes apoptosis through a variety of intermediates, and could therefore disrupt cellular physiology if a temperature threshold is exceeded (Circu and Aw, 2010; Hahm *et al.*, 2011; Matés and Sánchez-Jiménez, 2012). In the future we would like to conduct cell cycle analysis to determine at what temperature the celomocytes are reaching an apoptotic threshold perhaps mediated by a p53-like homolog, and determine if these cells can mediate a DDR-like mechanism. In addition to its role in host defense mechanisms, ROS has been shown to be involved in many other cellular processes including proliferation, growth arrest, as well as the initiation of apoptosis (Liu *et al.*, 2008). It would be of interest to determine if ROS participate in these cellular processes in the earthworm. At this stage it is unclear whether heat stress in earthworm celomocytes induces ROS production that culminates in DDR or vice versa.

It is clear from the results of our study that there is a correlation between increased ROS production and cell death as invertebrate celomocytes are subjected to heat stress. Therefore, as ROS begin to accumulate they have a negative effect on cellular viability and physiological processes. Evidence indicates that ROS-mediated damage predisposes complex organisms to cancer (Pelicano *et al.*, 2004). Although cancer has not been observed in the earthworm, ROS-mediated damage can result in impaired physiological function through cellular damage of DNA, proteins, lipids and other macromolecules (Rowe *et al.*, 2008). It is possible that heat-induced ROS can be generated in other organisms that share the soil environment through heat stress.

Through our flow cytometric analysis of ROS production, cell viability and histone phosphorylation we show that the earthworm has the potential to be used as a bioindicator to monitor heat-related environmental stressors such as climate change. Global warming has the potential to compromise soil ecosystems in the next thirty to sixty years by influencing biotic and abiotic factors, and may mediate habitat displacement of soil-dwelling invertebrates by reducing the resources available for immune defense mechanisms and reproduction (Houle *et al.*, 2012; Mandrioli, 2012).

Acknowledgments

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