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RESEARCH ARTICLE

A conserved role for the 20S proteasome and Nrf2 transcription factor in oxidative stress adaptation in mammals, *Caenorhabditis elegans* and *Drosophila melanogaster*

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SUMMARY

In mammalian cells, hydrogen peroxide (H_2O_2)-induced adaptation to oxidative stress is strongly dependent on an Nrf2 transcription factor-mediated increase in the 20S proteasome. Here, we report that both *Caenorhabditis elegans* nematode worms and *Drosophila melanogaster* fruit flies are also capable of adapting to oxidative stress with H_2O_2 pre-treatment. As in mammalian cells, this adaptive response in worms and flies involves an increase in proteolytic activity and increased expression of the 20S proteasome, but not of the 26S proteasome. We also found that the increase in 20S proteasome expression in both worms and flies, as in mammalian cells, is important for the adaptive response, and that it is mediated by the SKN-1 and CNC-C orthologs of the mammalian Nrf2 transcription factor, respectively. These studies demonstrate that stress mechanisms operative in cell culture also apply in disparate intact organisms across a wide biological diversity.

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INTRODUCTION

Cells, small organisms and animals frequently experience protein damage as a result of oxidative stress. The levels of oxidative stress to which all are exposed is not static, but varies based on both external factors, such as air pollution (Halliwell et al., 1992; Menzel, 1994), pesticides (Abdollahi et al., 2004), ozone (Cross et al., 1992a; Cross et al., 1992b), exposure to radiation including UV (Hu and Tappel, 1992) and ionizing radiation (Leach et al., 2001), and internal factors, such as mitochondrial activity (Kappus, 1987) and immune responses (Baeuerle et al., 1996). Although oxidative stress exposure is variable, the ability of cells and organisms to cope with such stress can also be transiently varied to meet changing needs; this process is called oxidative stress adaptation (Davies, 2000; Pickering et al., 2010; Pickering et al., 2012; Wiese et al., 1995), or conditioning hormesis (Gems and Partridge, 2008; Ristow and Zarse, 2010). In previous studies using cultured mammalian cells, oxidative stress adaptation has typically been assayed using a pre-treatment and challenge model; in this model, exposing cells to a mild oxidative stress produces a transient increase in tolerance to a subsequent higher (and normally toxic) stress (Davies, 2000; Pickering et al., 2010; Pickering et al., 2012; Wiese et al., 1995). Such oxidative stress adaptation lasts for no more than 48 h (if the stimulating oxidant is removed or metabolized) and involves direct enzyme activation, upregulated expression of more than 50 protective and transient growth-arrest genes, and downregulation of a similar number of housekeeping and proliferative genes.

In mammalian cell culture (murine embryonic fibroblasts), an important part of this adaptive response is an increase in the 20S proteasome. Blocking the induction of 20S proteasome expression [with small interfering RNA (siRNA) or antisense RNA] significantly limits the effectiveness of the adaptive response (Pickering et al., 2010). The induction of the 20S proteosome is regulated by the stress-responsive transcription factor Nrf2 [nuclear factor (erythroid-derived 2)-like 2] (Pickering et al., 2012). In mammalian cells, adaptive increases in the levels and activity of the 20S proteasome, as well as the Pa28αβ (or 11S) proteasome regulator and the immunoproteasome, play a vital role in the proteolytic removal of oxidatively damaged proteins. This enables cells to cope with oxidative stress, and permits normal cell function to continue (Crawford et al., 1996; Davies, 1993; Davies, 2000; Pickering et al., 2010; Wiese et al., 1995).

Whole-organismal adaptation to oxidative stress has been documented in the nematode worm *Caenorhabditis elegans*. Exposure of the worm to a mild heat shock, or a mild dose of an oxidant (such as brief exposure to 100% oxygen or the quinone juglone), results in increased tolerance to a future, more severe, heat or oxidant assault. Interestingly, such adaptation can also result in an increased lifespan (Cypser and Johnson, 2002; Lithgow et al., 1995; Przybysz et al., 2009). Similarly, in the common fruit fly, *Drosophila melanogaster*, a mild oxidant pre-treatment, or irradiation, increases the tolerance of the fly to a future, normally toxic oxidant challenge (Moskalev et al., 2009). In the present study, experiments were conducted to test whether transient adaptation to hydrogen peroxide (H_2O_2) in *C. elegans* and *D. melanogaster* follows the same precepts that operate in mammalian cells in culture, and whether adaptation in worms and flies also depends on induction of the 20S proteasome, and if this induction is regulated by (orthologs of) the Nrf2 transcription factor. Although Pa28αβ and immunoproteasome are important for adaptation in mammalian cells (Pickering et al., 2010), neither is present in *C. elegans* or *D.*

melanogaster, and while both organisms have a ortholog of the Pa28γ regulator, its function remains largely unclear (Rechsteiner and Hill, 2005). For these reasons, attention was focused on the 20S proteasome and Nrf2 in worms and flies.

MATERIALS AND METHODS

Caenorhabditis elegans culture

Strains were maintained at 20°C as described by Brenner (Brenner, 1974). A bacterial lawn of OP50 *Escherichia coli* was spread on the plates for feeding. The N2 Bristol strain was used as the control for all experiments. *skn-1(zu67)* was obtained from the *Caenorhabditis* Genetics Center, University of Minnesota. For experiments investigating SKN-1::GFP localization, the strain LD1 containing the integrant idIs7, generously provided by the Blackwell lab (Harvard Medical School, Boston, MA, USA), was crossed into *glo-1(zu391)* to minimize auto-fluorescence. M9 solution (35 mmol l^{-1} Na_2HPO_4, 22 mmol l^{-1} KH_2PO_4, 85 mmol l^{-1} NaCl, 19 mmol l^{-1} NH_4Cl) was used as media for washing worms and for treating worms with H_2O_2.

Preparation of age-matched *C. elegans* cultures

In all experiments age-matched worm cultures were used. It was found that egg preparation using an NaOH/bleach solution, as is commonly used for age synchronization (Stiernagle, 2006), itself produced a stress response. Therefore, eggs layed over a 24 h period were used for synchronization.

Caenorhabditis elegans H_2O_2 adaptation

Two to three day old (juvenile/young adult) worms were collected and washed with M9 medium; worms were washed 3 times to remove any contamination from the bacterial lawn. Worms were then transferred to Eppendorf tubes containing 1 ml of M9 medium, plus 0, 0.1, 1 or 10 μmol l^{-1} H_2O_2. Worms were incubated in this medium with mild shaking for 1 h then returned to normal plates for 24 h to permit adaptation to occur. Unless otherwise stated, 1 μmol l^{-1} H_2O_2 was used as an adaptive pre-treatment for *C. elegans*.

Caenorhabditis elegans H_2O_2 challenge

Three to four day old adult worms were collected and washed with M9 medium; worms were washed 3 times to remove any contamination from the bacterial lawn. Worms were then transferred to Eppendorf tubes containing 1 ml of M9 medium plus 100 μmol l^{-1} to 160 mmol l^{-1} H_2O_2. Worms were incubated in this medium, with mild shaking, for 1 h then returned to normal plates. Survival was either scored instantly or 24 h later, by response to prodding with a pick. This experiment was typically performed blind, by another member of the group encoding and randomizing the plates immediately before they were scored. Where pre-treatment challenge assays were performed, pre-treatment was typically carried out 24 h prior to challenge and 80 mmol l^{-1} H_2O_2 was used for the challenge dose unless otherwise stated.

SKN-1 green fluorescent protein (GFP) worm imaging

Age-matched idIs7;*glo-1(zu391)* worms were prepared as described above. L4 animals were used for imaging experiments as they have lower levels of intestinal auto-fluorescence. Animals were collected 48 h after egg laying and washed 3 times with M9 medium to remove any bacterial contamination. Worms were then transferred to Eppendorf tubes containing 1 ml of M9 medium ±1 μmol l^{-1} H_2O_2 or ±10 mmol l^{-1} H_2O_2. Worms were incubated ±H_2O_2 for 20 min and then paralyzed by the addition of 2,3-butanedione monoxime. Worms were then mounted on slides containing an agarose pad. SKN-1::GFP nuclear localization was scored by microscopic analysis using a 500–1000× magnification with GFP illumination (An and Blackwell, 2003; An et al., 2005).

Drosophila melanogaster culture

Drosophila melanogaster were cultured on a standard agar/dextrose/corn meal/yeast media (Ren et al., 2009) at 25°C. Unless otherwise stated, w[1118] flies were used in all assays. Flies were collected over a 48 h period from pre-cleared bottles, and allowed 4 days to mature so that at initiation of assays, flies were 4–6 days old.

Drosophila H_2O_2 challenge assays

Samples of 20 flies were transferred to vials containing half a Kimwipe soaked in 1 ml of 5% sucrose and 4.4 mol l^{-1} H_2O_2. Survival was then scored ever 6 or 8 h following initiation of the challenge. Flies were scored as dead once they became completely immobile.

Drosophila H_2O_2 adaptation

Samples of 20 flies were transferred to vials containing half a Kimwipe soaked in 1 ml of 5% sucrose plus 0, 10 or 100 μmol l^{-1} or 1 mmol l^{-1} H_2O_2 for 8 h. Flies were then returned to normal vials for 16 h to permit adaptation to occur. Flies were then challenged with a toxic dose of H_2O_2. In the case of experiments using *cnc-C*^{RNAi} flies, we found that, for effective adaptation to occur, flies needed to be incubated on H_2O_2-soaked Kimwipes for 24 h instead of 8 h. In addition, because of the longer incubation time in the experiments with *cnc-C*^{RNAi} flies, 10 μl of either ethanol or RU-486 was added to the Kimwipes during this treatment.

Western blot assays

Preparation of *C. elegans*

Approximately 1000–2000, 3–4 day old adult worms were collected per sample and washed with M9 medium. Worms were washed 3 times with M9 medium to remove any bacterial contamination. Worms were then transferred into a solution containing RIPA buffer (catalog no. 89901, Thermo Fisher, Waltham, MA, USA), supplemented with protease inhibitor cocktail (catalog no. 11836170001, Roche, Nutley, NJ, USA). We then lysed the worms by freezing them once, and subjecting them to 3×10 s bursts of sonication, followed by 3 cycles of incubation on ice for 5 min, and vortexing. Lysates were centrifuged at 10,000***g*** to remove un-lysed fragments. Protein content was quantified with a BCA protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions for western analysis.

Preparation of *Drosophila*

Twenty flies were collected per sample. Flies were then transferred into a solution containing RIPA buffer supplemented with protease inhibitor cocktail, as above. Flies were frozen, then homogenized using an electronic pestle after which lysis was performed by 3×5 min incubations on ice, followed by vortexing. Samples were then centrifuged at 10,000***g*** to remove cuticle fragments and unlysed cells. Protein content was next quantified with the BCA Protein Assay Kit as for *C. elegans*.

Western blot analysis

A 40 μg sample of protein was run on an SDS-PAGE gel and transferred to a PVDF membrane using standard western blot techniques.

Fluorpeptide proteolytic assays

Preparation of *C. elegans*

Two-hundred adult worms were collected per sample and washed 3 times with M9 medium to remove bacterial contamination. Worms were then transferred into a solution containing proteolysis buffer (50 mmol l^{-1} Tris, 25 mmol l^{-1} KCl, 10 mmol l^{-1} NaCl, 1 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} DTT, pH 7.5). Worms were frozen once, then homogenized, after which cell lysis was achieved by three freeze–thaw cycles in dry ice for 5 min, followed by a room temperature water bath for 5 min. After this, samples were centrifuged at 10,000 ***g*** to remove cuticle fragments and unlysed cells. Protein content was quantified with the BCA Protein Assay Kit (Pierce) according to the manufacturer's instructions. Samples were then diluted to the appropriate concentration based on BCA assays results.

Preparation of *Drosophila*

Ten flies were collected per sample. Flies were then transferred into a solution containing proteolysis buffer (50 mmol l^{-1} Tris, 25 mmol l^{-1} KCl, 10 mmol l^{-1} NaCl, 1 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} DTT, pH 7.5). Flies were frozen once, then homogenized using a pestle, after which lysis was performed by three freeze–thaw cycles performed through incubation on dry ice for 5 min, followed by a room temperature water bath for 5 min. After this, samples were centrifuged at 10,000 ***g*** to remove cuticle fragments and unlysed cells. Protein content was quantified with the BCA Protein Assay Kit (Pierce) according to the manufacturer's instructions. Samples were then diluted to the appropriate concentration based on BCA assays results.

Fluorpeptide proteolytic analysis

Aliquots of 5.0 µg to 1 mg (depending on the assay) were transferred, in triplicate, to 96-well plates, and 2 µmol l^{-1} of *N*-succinyl-Leu-Leu-Val-Tyr-AMC (catalog no. 80053-860, VWR, Chester, PA, USA) was added to each well. Plates were incubated at 37°C and mixed at 300 r.p.m. for 4 h. Fluorescence readings were taken at 10 min intervals using an excitation wavelength of 355 nm and an emission wavelength of 444 nm. Fluorescence units were converted to moles of free 7-amino-4-methylcoumarin (AMC), with reference to an AMC standard curve of known amounts of AMC (catalog no. 164545, Merck, Whitehouse Station, NJ, USA), following subtraction of background fluorescence. In some experiments, cells were treated with 20 µmol l^{-1} of the proteasome inhibitor lactacystin (catalog no. 80052-806, VWR), 30 min prior to incubation and addition of substrates. Lactacystin was dissolved in DMSO at a 100× concentration and combined with samples at a concentration of 0.1%.

Caenorhabditis elegans RNA intereference (RNAi) treatment

Two to three day old (juvenile/young adult) worms were cultured on plates containing a bacterial lawn expressing RNAi against the proteasome subunits *pas-5*, *pbs-3*, *pbs-5* and *pbs-6*. In addition, some plates contained bacteria expressing RNAi against *skn-1* or an empty vector control (*L4444*). Worms were incubated on these plates for 6 h, and then collected and washed 3 times with M9 medium to remove any contamination from the bacterial lawn. Worms were then transferred to Eppendorf tubes containing 1 ml of M9 medium ±1 µmol l^{-1} H_2O_2. Worms were incubated, with mild shaking, for 1 h then returned to RNAi plates for 24 h. Worms were collected and washed 3 times with M9 medium to remove any bacterial contamination. Worms were then transferred to 48-well plates containing 500 µl of M9 medium + 8 mmol l^{-1} H_2O_2. Worms were incubated, with mild shaking, for 1 h, then survival was scored immediately (as above).

Drosophila RNAi experiments

Flies expressing RNAi against two proteasome subunits were purchased from the Vienna *Drosophila* RNAi center (VDRC, Vienna, Austria). These were *prosβ1*^{RNAi} (w[1118]; P[GD13913]v35923), and *prosβ2*^{RNAi} (w[1118]; P[GD10938]v24749). In addition we received the kind donation of flies expressing RNAi against *D. melanogaster* cap 'n' collar transcription factor (an ortholog of mammalian Nrf2; *cnc-C*) and *dkeap-1* from Dr Dirk Bohman (Sykiotis and Bohmann, 2008; Sykiotis and Bohmann, 2010). Males from these lines (or w[1118] as a control) were crossed with virgin females containing the Act-GS-255B driver (Fischer et al., 1988; Ford et al., 2007). Parents were removed 4 days after initiation of the cross. Progeny were then collected over a 48 h period after eclusion. The exception to this was the *cnc-C*^{RNAi} line where a low birthrate required collections over a 72 h period. The Act-GS-255B driver is activated by feeding flies RU-486. Flies were cultured in normal vials containing either 50 µl of stock RU-486 (20 mg ml^{-1}) or ethanol, which had been added to vials and air dried 24 h prior to the assay. Flies were incubated in these vials for 5–7 days. In experiments involving H_2O_2 adaptation, flies were removed from ±RU-486 vials 24 h before the end of RU-486 treatment. Flies were then transferred to vials containing half a Kimwipe soaked in 1 ml of 5% sucrose ±100 µmol l^{-1} H_2O_2 for 8 h. The flies were then returned to RU-486 or ethanol vials for the remaining 16 h of RU-486 treatment.

RESULTS

H_2O_2-induced adaptation to oxidative stress in *C. elegans*

To determine whether *C. elegans* are capable of H_2O_2 oxidative stress adaptation, a pre-treatment/challenge assay, similar to that developed in mammalian culture, was employed (Pickering et al., 2010; Pickering et al., 2012; Wiese et al., 1995). In this assay, age-matched young adult worms were exposed to low adaptive doses of H_2O_2 for 1 h, and 1 h later were assayed for survival in response to toxic doses of H_2O_2 (40–160 mmol l^{-1}) using standard survival assays (see Materials and methods) (Larsen, 1993). Worms that were not pre-treated exhibited a progressive decline in survival with increasing H_2O_2 challenge (Fig. 1A). A challenge dose of 80 mmol l^{-1} produced a 70% loss (only 30% survival) of non-pre-treated worms. Survival was increased to 45–50% with H_2O_2 pre-treatment (Fig. 1B). From the above results it appeared that mild H_2O_2 pre-treatment produces the expected enhanced resistance to oxidative stress in *C. elegans*.

The challenge doses used in the above experiment, however, were highly toxic and produced 0% survival 24 h after treatment, even in pre-treated samples (Fig. 1A; data not shown). To better test oxidative stress adaptation, it seemed reasonable to examine longer-term survival with less severe H_2O_2 challenges. To test this, the assays in Fig. 1A were repeated, using milder H_2O_2 challenge doses (200 µmol l^{-1} to 3.2 mmol l^{-1}). A 24 h recovery period was then permitted after H_2O_2 challenge before scoring survival. Using this protocol, a similar pattern of declining survival with increasing H_2O_2 challenge was observed (Fig. 1C). It was found that a 1.6 mmol l^{-1} H_2O_2 pre-treatment would produce a 16% survival increase upon subsequent challenge, and survival upon challenge was increased to over 30% with a 10 µmol l^{-1} H_2O_2 pre-treatment (Fig. 1D).

It is difficult to directly measure the activity of individual proteases in an extract, so instead their activity is approximated though the capacity of the extract to degrade peptide substrates ('proteolytic capacity'). It has previously been shown that an

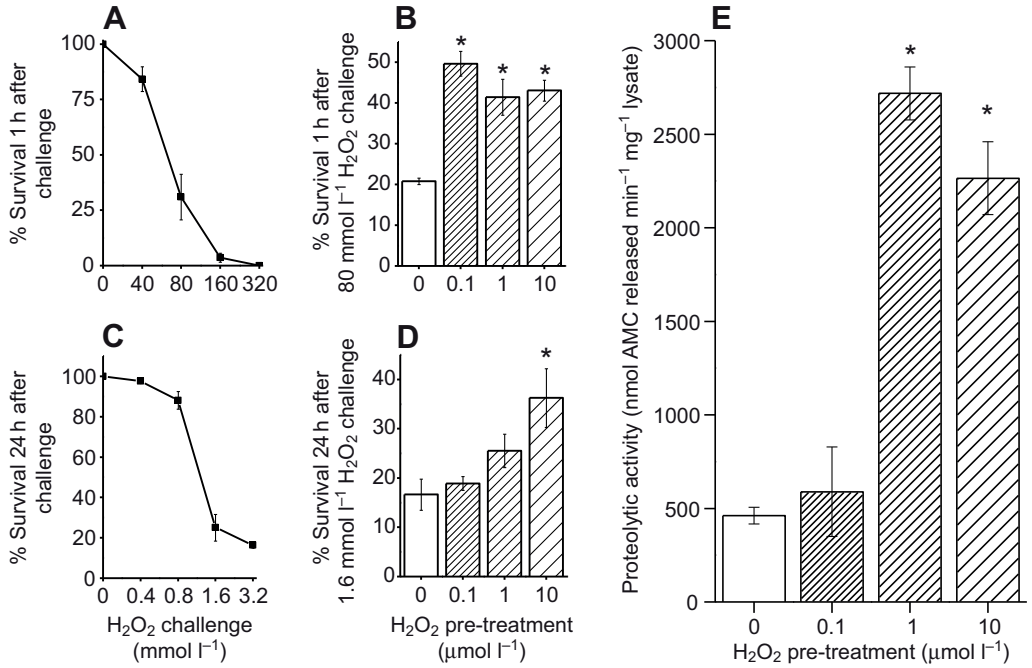

Fig. 1. (A) H_2O_2 challenge causes a decline in *Caenorhabditis elegans* survival 1 h after the challenge. Triplicate samples of 35–100 (typically ~70) age-matched, adult, N2 worms were challenged with the indicated concentrations of H_2O_2 for 1 h. Survival was scored immediately after the challenge. (B) H_2O_2 pre-treatment reduces H_2O_2 challenge-induced mortality in the first hour after challenge. Worms were pre-treated with the indicated concentrations of H_2O_2 then, 24 h later, challenged with 80 mmol l^{-1} H_2O_2. Survival was scored immediately after the challenge. (C) H_2O_2 challenge causes a decline in worm survival 24 h after the challenge. Triplicate samples of 40–195 (typically ~100) age-matched, adult, N2 worms were prepared and pre-treated as in A, using the indicated concentrations of H_2O_2, and survival was scored 24 h after challenge. (D) H_2O_2 pre-treatment reduces H_2O_2 challenge-induced mortality in the 24 h after challenge. Worms were pre-treated with the indicated concentrations of H_2O_2 then challenged, 24 h later, with 1.6 mmol l^{-1} H_2O_2. Survival was scored 24 h after the challenge. (E) H_2O_2 pre-treatment increases proteolytic capacity. Worms were treated with the indicated concentrations of H_2O_2 and lysed 24 h later. Proteolytic activity assays (degradation of the short peptide substrate Suc-LLVY-AMC) were performed on these samples as described in Materials and methods. In all cases, values are plotted as means ± s.e.m., N=3. Values marked with an asterisk indicate statistically significant differences ($P \leq 0.05$) using Student's t-test.

increase in proteolytic capacity is an important component of the adaptive response to oxidative stress in mammalian cell culture (Grune et al., 2011; Pickering et al., 2010; Pickering et al., 2012). To determine whether a similar increase in proteolytic capacity occurs in *C. elegans*, the proteolytic activities of whole-worm extracts were examined 24 h after pre-treating worms with H_2O_2 (as per Fig. 1B,D), by measuring degradation of the short peptide substrate Suc-LLVY-AMC. H_2O_2 pre-treatment produced a 5-fold adaptive increase in proteolytic capacity in extracts from animals that were pre-treated with mild H_2O_2 compared with those extracts that were not pre-treated (Fig. 1E). Interestingly, pre-treatment with either 1 or 10 μmol l^{-1} H_2O_2 increased both survival and proteolytic activity, whereas 0.1 μmol l^{-1} pre-treatment did not significantly increase survival or proteolytic activity. This appears to be a non-linear response in which a mild H_2O_2 (0.1 μmol l^{-1}) pre-treatment yields a weak increase in proteolytic activity but a much stronger increase in oxidative stress tolerance.

Role of the 20S proteasome in H_2O_2-induced adaptation in *C. elegans*

These results suggest that as an overall phenomenon, oxidative stress adaptation and the corresponding increase in proteolytic activity are similar in *C. elegans* to that observed in mammalian cells (Grune et al., 2011; Pickering et al., 2010; Wiese et al., 1995). In the mammalian system, upregulation of the 20S proteasome has been shown to play an important role in this response through an increase in the capacity of cells to degrade oxidized proteins (Pickering et al., 2010; Pickering et al., 2012). To see whether this was also the case in *C. elegans*, the level of 20S proteasome protein under H_2O_2 adaptation was measured. To do this, animals were pre-treated with a variety of mild H_2O_2 doses. Subsequently, worm lysates from these pre-treated animals were analyzed for the abundance of either the 20S proteasome subunit PAS-7 or the 19S regulator subunit RPN-10 (proteasome regulatory particle non-ATPase-like 10, a key component of the 26S proteasome) using western blotting (Fig. 2A,B). This H_2O_2 pre-treatment produced a 2- to 3-fold increase in 20S proteasome (PAS-7) levels with no significant change in the levels of the 19S regulator of the 26S proteasome (RPN-10). These results suggest that the 20S but not the 26S proteasome may be involved in oxidative stress adaptation.

Because the 20S proteasome was induced by H_2O_2 treatment it was next determined whether 20S proteasome induction is important for the adaptive response. To test this, conditioning hormesis was assayed in animals in which the 20S proteasome was first knocked-down by RNAi. The effects of knock-down of four 20S subunit genes were tested. These corresponding RNAi clones were available from an RNAi library (Kamath et al., 2003): *pas-5*, *pbs-3*, *pbs-5* or *pbs-6*. In this experiment, worms were cultured with RNAi directed against the 20S proteasome subunits *pas-5*, *pbs-3*, *pbs-5* or *pbs-6*, or the empty vector (*L4440*) for 6 h to block new synthesis of proteasome genes without significantly reducing baselines levels. Worms were then transferred into 1 μmol l^{-1} H_2O_2 for 1 h to induce

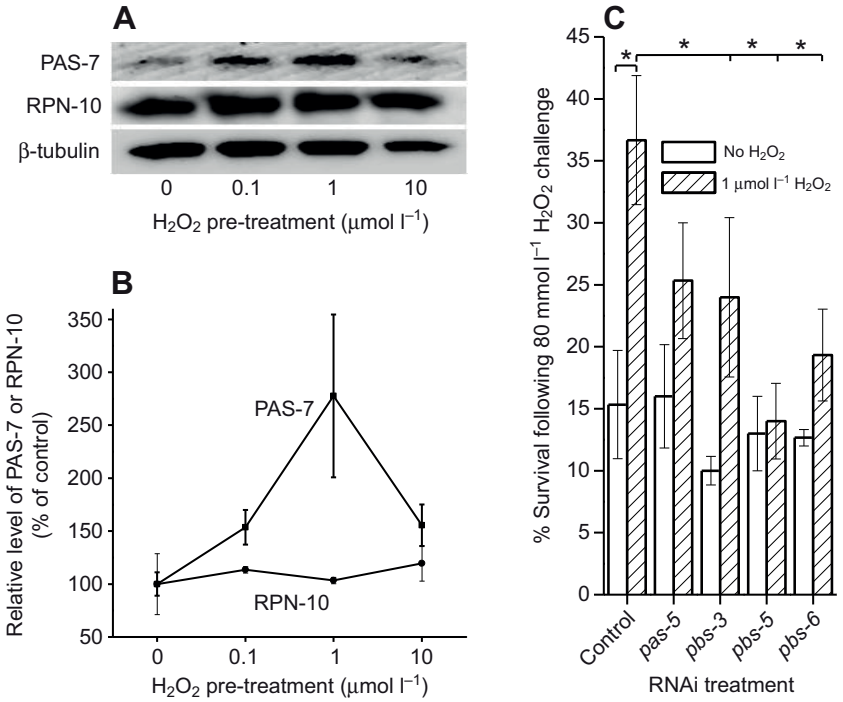

Fig. 2. (A) H_2O_2 pre-treatment causes an adaptive increase in the expression of the 20S proteasome (PAS-7) but not the 26S proteasome (19S regulator RPN-10). Triplicate samples of N2 worms were pre-treated with the indicated concentrations of H_2O_2. Worms were then lysed 24 h later and the lysate was analyzed by western blotting. (B) Samples were prepared as in A, in triplicate. Values are plotted as means (normalized to β-tubulin) ± s.e.m., *N*=3. (C) Blocking the H_2O_2-induced adaptive increase in the 20S proteasome blunts the pre-treatment-induced increase in oxidative stress tolerance. Worms were cultured on the indicated RNA interference (RNAi) plates for 6 h, then pre-treated with 1 μmol l^{-1} H_2O_2 for 1 h, after which worms were returned to RNAi plates for 24 h. Worms were then challenged with H_2O_2 and survival was scored. All values are means ± s.e.m., *N*=3. Values marked with an asterisk indicate statistically significant differences ($P \leq 0.05$) using Student's *t*-test.

adaptation. Worms were subsequently returned to their RNAi plates for a further 24 h, and then collected for adaption experiments, performed as in Fig. 1B. RNAi knockdown of the four proteasomal subunits did not significantly alter survival on 80 mmol l^{-1} H_2O_2 compared with control RNAi treatments; however, RNAi knockdown of any one of these proteasome subunits severely blunted the enhanced survival of animals that were pre-treated with adaptive doses of H_2O_2 (Fig. 2C). RNAi knockdown of *pbs-5* appeared to be the most effective, whereas *pas-5*, *pbs-3* and *pbs-6* appeared to have partial effects. This may be due to differences in the efficacy of RNAi in these experiments or it may reflect differences in the functions of these subunits in the proteasome. These results indicate that the 20S proteasome plays an important role in the adaptive increase in oxidative stress tolerance exhibited by H_2O_2 pre-treated *C. elegans*.

The role of SKN-1 in oxidative stress adaptation in *C. elegans*

Nrf2 has previously been found to be an important regulator of oxidative stress adaptation in mammalian cell culture (Pickering et al., 2012). At least part of the reason for this is the Nrf2-dependent increase in 20S proteasome synthesis during mammalian cell oxidative stress adaptation (Pickering et al., 2012). Studies by the Blackwell lab have demonstrated that SKN-1 is a functional ortholog of Nrf2 (An and Blackwell, 2003). Like Nrf2, SKN-1 is normally maintained at low levels in the cytoplasm, but under conditions of oxidative stress it translocates to the nucleus (An and Blackwell, 2003; An et al., 2005; Kahn et al., 2008; Oliveira et al., 2009). It has been shown that high doses of H_2O_2 (10 mmol l^{-1}) can cause the cytoplasmic to nuclear translocation of SKN-1 fused to GFP (SKN-1::GFP) in *C. elegans* intestinal cells (An and Blackwell, 2003; An et al., 2005). Also, a report by Przybysz and colleagues has shown that with depletion of SKN-1, worms become more susceptible to the oxidant juglone (Przybysz et al., 2009). The SKN-1::GFP line (An and Blackwell, 2003), was crossed with a *glo-1* mutant strain to produce worms with reduced non-specific fluorescence. Using transgenic animals expressing the SKN-1::GFP reporter, the number of fluorescent SKN-1::GFP intestinal nuclei in H_2O_2-treated animals was compared with that in untreated controls. First, it was confirmed that exposure to 10 mmol l^{-1} H_2O_2 caused a 3-fold increase in the number of SKN-1::GFP fluorescent nuclei (Fig. 3A,B). Treated worms had an average of 10.0±1.4 nuclei exhibiting expression of GFP, compared with 3.7±1.2 in untreated worms (Fig. 3B). It was next tested whether lower adaptive treatment concentrations could also produce an increase in SKN-1::GFP nuclear localization. The experiments of Fig. 3A,B were repeated, using an adaptive dose of 1 μmol l^{-1} H_2O_2, and a smaller but still significant increase in the number of SKN-1::GFP fluorescent nuclei was observed relative to untreated controls (Fig. 3C,D). Treated worms had an average of 6.1±1.1 nuclei exhibiting expression of GFP, compared with 2.1±0.6 in untreated worms (Fig. 3B). These results suggest that low adaptive doses of H_2O_2 that confer enhanced survival may activate SKN-1 by promoting its translocation to the nucleus.

To determine whether SKN-1 is important in H_2O_2-induced oxidative stress adaptation, experiments were performed to test whether enhanced survival following pre-treatment through H_2O_2 adaptation is dependent on the presence of SKN-1. Knockdown of SKN-1 by RNAi did not alter survival of non-pre-treated animals compared with control RNAi-treated animals upon challenge with 80 mmol l^{-1} H_2O_2. However, the adaptive response with 1 μmol l^{-1} H_2O_2 pre-treatment was blunted following treatment with RNAi against *skn-1* (Fig. 3E). Thus, not only does the adaptive response in *C. elegans* involve an increase in SKN-1 nuclear localization but also SKN-1 activity is important for oxidative stress adaptation, implicating SKN-1 nuclear translocation as a crucial event in oxidative stress adaptation. However, as it was unclear how effective the SKN-1 RNAi was at causing depletion of *skn-1*, the experiment was repeated using a *skn-1(zu67)* mutant (Bowerman et al., 1992). The *skn-1(zu67)* mutant encodes an early stop codon that is predicted to eliminate activity of the two SKN-1 isoforms (SKN-1a and SKN-1c) that are expressed in the intestine and are required for stress responses (An and Blackwell, 2003). Despite pre-treatment of this mutant line with a range of doses of H_2O_2, no adaptive increase in oxidative stress tolerance was observed upon H_2O_2 challenge

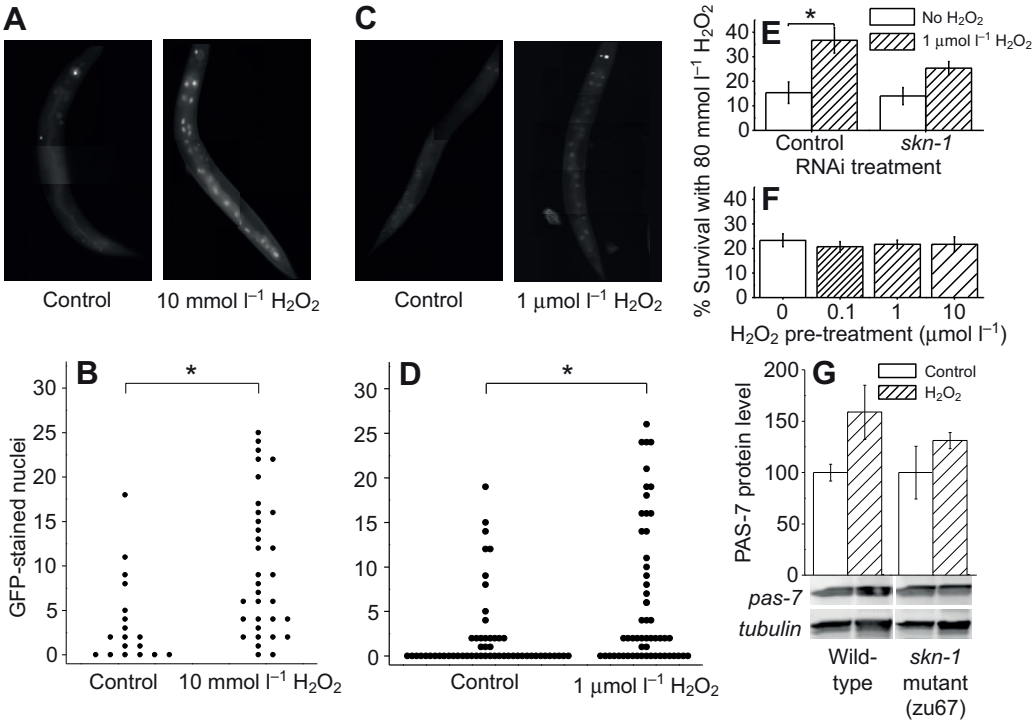

Fig. 3. (A) Pre-treatment with 10 mmol l^{-1} H_2O_2 causes an adaptive increase in SKN-1 nuclear localization. Representative images of L4 stage worms expressing an SKN-1::GFP transgene (idIS7) in intestinal nuclei following treatment with 10 mmol l^{-1} H_2O_2. (B) Quantification of the number of fluorescent nuclei observed per worm following 10 mmol l^{-1} H_2O_2 treatment (*N*=20–30 worms). (C) Treatment with 1 μmol l^{-1} H_2O_2 also causes an adaptive increase in SKN-1::GFP nuclear fluorescence. Worms were prepared as in A, except worms were treated with 1 μmol l^{-1} H_2O_2. (D) Quantification of the number of fluorescent nuclei observed per worm following 1 μmol l^{-1} H_2O_2 treatment (*N*=50–60 worms). (E) RNAi knockdown of SKN-1 blunts the pre-treatment-induced adaptive increase in oxidative stress tolerance. Percentage survival of either control or *skn-1* RNAi-treated worms was measured following H_2O_2 pre-treatment and challenge as described in Materials and methods. (F) *skn-1(zu67)* mutants do not appear to have an H_2O_2 pre-treatment-induced adaptive increase in oxidative stress tolerance. Adult *skn-1* mutant worms were pre-treated and subsequently challenged with H_2O_2 as described in Materials and methods. (G) H_2O_2 pre-treatment causes a *skn-1*-dependent adaptive increase in 20S subunit protein abundance, which is blunted in *skn-1(zu67)* mutants. Shown are representative western blots and quantification of PAS-7 protein abundance in wild-type or *skn-1* mutants 24 h after pre-treatment with 1 μmol l^{-1} H_2O_2 and in controls. Values are plotted as means (normalized to tubulin) ± s.e.m., *N*=3. Values marked with an asterisk indicate statistically significant differences ($P \leq 0.05$) compared with controls using Student's *t*-test.

(Fig. 3F). This result is therefore highly supportive of an important role for *skn-1* in regulating the adaptive response.

Given the role of mammalian Nrf2 in regulating proteasome function (Pickering et al., 2012), it is possible that SKN-1 may also regulate proteasome activity in response to adaptive doses of H_2O_2. This idea is consistent with the finding that at least half of the proteasome subunit genes in the *C. elegans* genome contain SKN-1 binding sites and are inducible by SKN-1 (Oliveira et al., 2009). Furthermore, it has been demonstrated that RNAi depletion of SKN-1 causes a marked reduction in the proteolytic capacity of the worm (Kirkwood, 2005). To determine whether SKN-1 regulates the oxidative stress-induced adaptive increase in the 20S proteasome, western blotting was used to measure the levels of a 20S proteasome subunit, PAS-7, following 1 μmol l^{-1} H_2O_2 pre-treatment, in wild-type (N2) worms and *skn-1(zu67)* mutant worms. H_2O_2 pre-treatment of control animals caused a 60% increase in the level of PAS-7 from whole-worm lysates (Fig. 3G). In contrast, only a 30% increase in PAS-7 levels was observed following H_2O_2 treatment in lysates from the *skn-1* mutant animals (Fig. 3G), and PAS-7 is one of the subunits previously shown to be inducible by *skn-1* (Oliveira et al., 2009). These results suggest that *skn-1* regulates the oxidative stress-induced increase in the 20S proteasome in *C. elegans*, similar to the effect of Nrf2 in mammalian cell cultures.

H_2O_2-induced increase in proteolytic capacity in *D. melanogaster*

Previous studies have shown that pre-treatment of *D. melanogaster* with mild doses of oxidants or radiation can result in a subsequent increase in tolerance to oxidative damage (Moskalev et al., 2009). First, we investigated whether H_2O_2 pre-treatment in *D. melanogaster* could be used to induce an increase in proteolytic capacity as has been seen in other model systems. Because of the greater size and complexity of the fly compared with mammalian cell cultures, and even nematode worms, it was not possible to simply incubate flies in H_2O_2. Instead, flies were maintained in vials without standard food, containing a Kimwipe soaked in 5% sucrose with or without an adaptive dose of H_2O_2 (Grover et al., 2009). For these experiments, flies were collected 0–48 h after eclosion. Flies were then allowed to mature for 4 days, after which they were transferred into vials containing Kimwipes soaked in 5% sucrose with or without 10–1000 μmol l^{-1} H_2O_2 for 8 h. It was confirmed that the sucrose/H_2O_2 solution was ingested by the flies over the 8 h incubation period by uptake of a visible dye (10% blue food color, Amerifoods, Los Angeles, CA, USA) (supplementary material Fig. S1). The flies were returned to normal vials for 16 h to permit adaptation, and then homogenized. The proteolytic capacity of the fly lysates was then measured by degradation of the fluorogenic

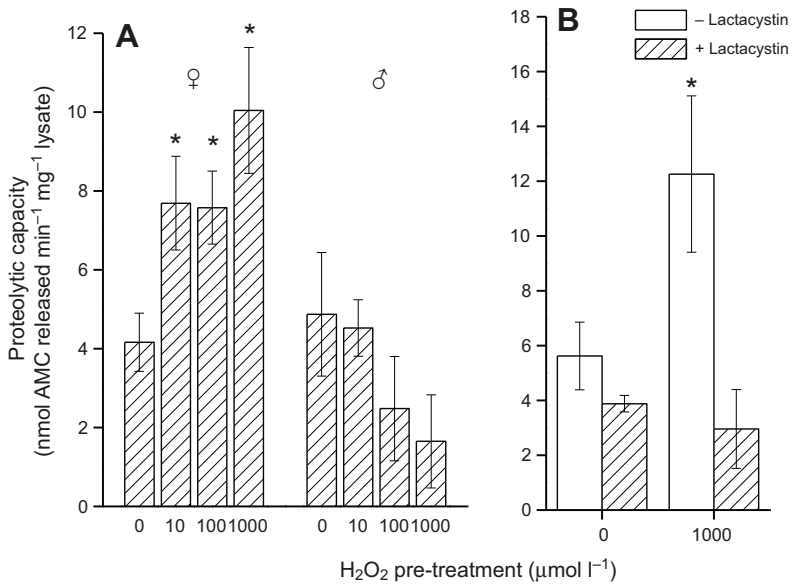

Fig. 4. (A) H_2O_2 treatment of *D. melanogaster* increases proteolytic capacity in females but not in males. Triplicate vials of w[1118] flies were pre-treated with the indicated concentrations of H_2O_2. Proteolytic activity assays were performed on flies 24 h later, measured as the degradation of Suc-LLVY-AMC (see Materials and methods). (B) The H_2O_2-induced adaptive increase in female fly proteolytic capacity is proteasome dependent. The control and 100 μmol l^{-1} H_2O_2-pre-treated samples from A were treated with the proteasome inhibitor lactasystin, 30 min prior to the addition of Suc-LLVY-AMC. In all cases, values are plotted as means ± s.e.m., *N*=3. Values marked with an asterisk indicate statistically significant differences ($P \leq 0.05$) using Student's *t*-test.

peptide model substrate Suc-LLVY-AMC. As shown in Fig. 4A, a progressive increase in proteolytic capacity was observed in females following H_2O_2 pre-treatment. In contrast, no increase in proteolytic capacity was observed when male flies were pre-treated with H_2O_2: instead, 100 and 1000 μmol l^{-1} H_2O_2 pre-treatment was found to decrease the proteolytic capacity of lysates from male flies (Fig. 4A).

This difference in adaptive response between male and female flies might be due to different food consumption rates. Female *Drosophila* consume approximately 3 times as much food as their male counterparts, have a much larger gut, and have more stem cells in their digestive tract (Wong et al., 2009). It was confirmed that during the 8 h H_2O_2 pre-treatment, male flies consumed considerably less of the sucrose/H_2O_2 solution than did female flies, and this might mean that male flies have a lower exposure to the adaptive dose of H_2O_2 and so are less responsive to H_2O_2 pre-treatment (supplementary material Fig. S1). Although it is clear that male flies consumed less than half as much of the sucrose/H_2O_2 solution as did females (see supplementary material Fig. S1), we can think of no reason why this difference in ingestion would negatively affect proteolytic capacity. An alternative hypothesis is that the observed differences are due to sexual dimorphism in stress responses between male and female flies and, consistent with this idea, sexual dimorphism has previously been observed in thermotolerance and oxidative stress resistance in *Drosophila* (Sørensen et al., 2007; Waskar et al., 2009; Weber et al., 2012).

To determine whether the increased proteolytic capacity of female flies (Fig. 4A) might be due to increased proteasome activity, selected samples were pre-exposed to the proteasome-selective inhibitor lactacystin, 30 min before proteolytic capacity was measured (Pickering et al., 2010). No increase in proteolytic capacity was evident in lactacystin-treated lysates, indicating that increased proteasome activity underlies the elevated proteolytic capacity (Fig. 4B).

H_2O_2-induced adaptation to oxidative stress in *D. melanogaster*

Because H_2O_2 treatment increased proteolytic capacity in female fly extracts, but not male fly extracts, it was next tested whether stress resistance and proteasome levels would correlate with tolerance to oxidative stress in an adaptation experiment. The first step was to develop an appropriately toxic challenge for the flies. Instead of subjecting flies to a single challenge as in mammalian cell culture and *C. elegans*, the assay employed for *D. melanogaster* involved incubating the flies in vials containing the toxic dose of H_2O_2 and scoring survival over time, as previously described (Grover et al., 2009; Sykiotis and Bohmann, 2008; Sykiotis and Bohmann, 2010). Survival was scored every 8 h, for up to 104 h. The survival curves for male and female flies cultured in vials containing Kimwipes with 0, 1, 2, 4 or 8 mol l^{-1} H_2O_2 are shown in supplementary material Fig. S2.

Having determined an optimal H_2O_2 challenge concentration of 4.4 mol l^{-1} (15%) (supplementary material Fig. S2), adaptation experiments were performed using flies prepared and pre-treated as in Fig. 4. Then, 24 h after initiating pre-treatment (16 h after terminating pre-treatment), flies were transferred to vials containing Kimwipes soaked in 5% sucrose and a toxic challenge dose of 4.4 mol l^{-1} (15%) H_2O_2. Survival was scored every 8 h, for up to 80 h. Non-pre-treated female flies survived an average of 48 h following initiation of the challenge. By comparison, female flies pre-treated with 100 μmol l^{-1} H_2O_2 (which was found to be the optimum pre-treatment) survived an average of 64 h. This represented a significant increase in survival time (Fig. 5A,B). In contrast, no change in survival time was seen following pre-treatment of male flies; both pre-treated and non-pre-treated male flies survived an average of 40 h following initiation of the challenge (Fig. 5A,B), thereby supporting a functional correlation between proteolytic activity and adaptation.

Role of the 20S proteasome in oxidative stress adaptation in *D. melanogaster*

Next, we tested whether 20S proteasome expression is induced by H_2O_2 treatment in female flies. To do this, female flies were pre-treated for 8 h with 100 μmol l^{-1} H_2O_2, which appeared to be the optimum treatment regime. Then, 24 h after the start of pre-treatment, female flies were homogenized and the lysates were analyzed by western blotting. The western blots were incubated with antibodies directed against the α-subunit of the 20S proteasome, the CG13349 subunit of the 19S regulator of the 26S proteasome, and β-tubulin as a loading control. As presented in Fig. 5C, a 50–60% increase in the expression of the 20S proteasome α-subunit was

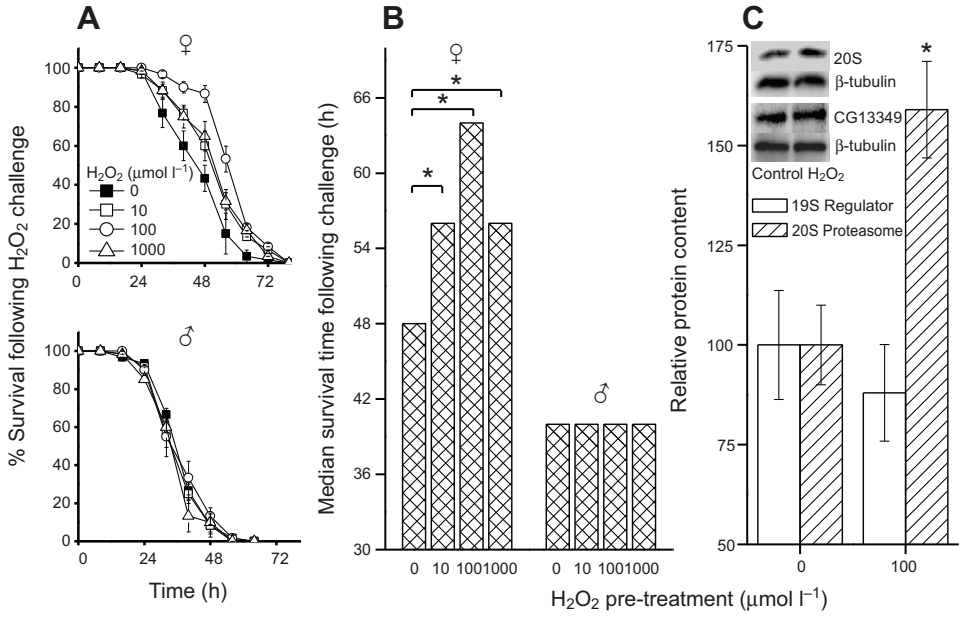

Fig. 5. (A) H_2O_2 pre-treatment enhances resistance to oxidative stress challenge. Triplicate vials of w[1118] flies were pre-treated with the indicated concentrations of H_2O_2 then challenged with 4.4 mol l^{-1} H_2O_2, 24 h later. Percentage survival was scored every 8 h based on complete loss of motion. (B) Median survival time for A (N=60 flies). (C) H_2O_2 pre-treatment induces increased expression of the 20S proteasome (α-subunit), but not of the 26S proteasome (19S regulator, CG13349 subunit). Triplicate vials of 20 w[1118] flies were pre-treated with 100 μmol l^{-1} H_2O_2 and western blots were run with samples 24 h later. The protein content was normalized to that of β-tubulin. Representative blots are shown in the inset. In all cases, values are plotted as means ± s.e.m., N=3. Values marked with an asterisk indicate statistically significant differences (P≤0.05) using a log-rank test in B and Student's t-test in C.

observed (reflecting increased overall 20S proteasome levels) but there was no change in the expression of the 19S regulator (indicating no increase in the 26S proteasome). Having seen an increase in the female fly 20S proteasome as a product of oxidative stress adaptation, we next asked whether this induction was required for adaptation to occur. To test this, two RNAi lines directed against the *prosβ1* and *prosβ2* 20S proteasome subunits were used. These lines were crossed to the Act-GS-255B strain (Ford et al., 2007) enabling the RNAi to be conditionally expressed in the presence of the drug RU-486 in all the somatic tissues of the adult fly (Shen et al., 2009). Additionally, the Act-GS-255B strain was crossed with w[1118] flies as controls for potential effects of RU-486. The female

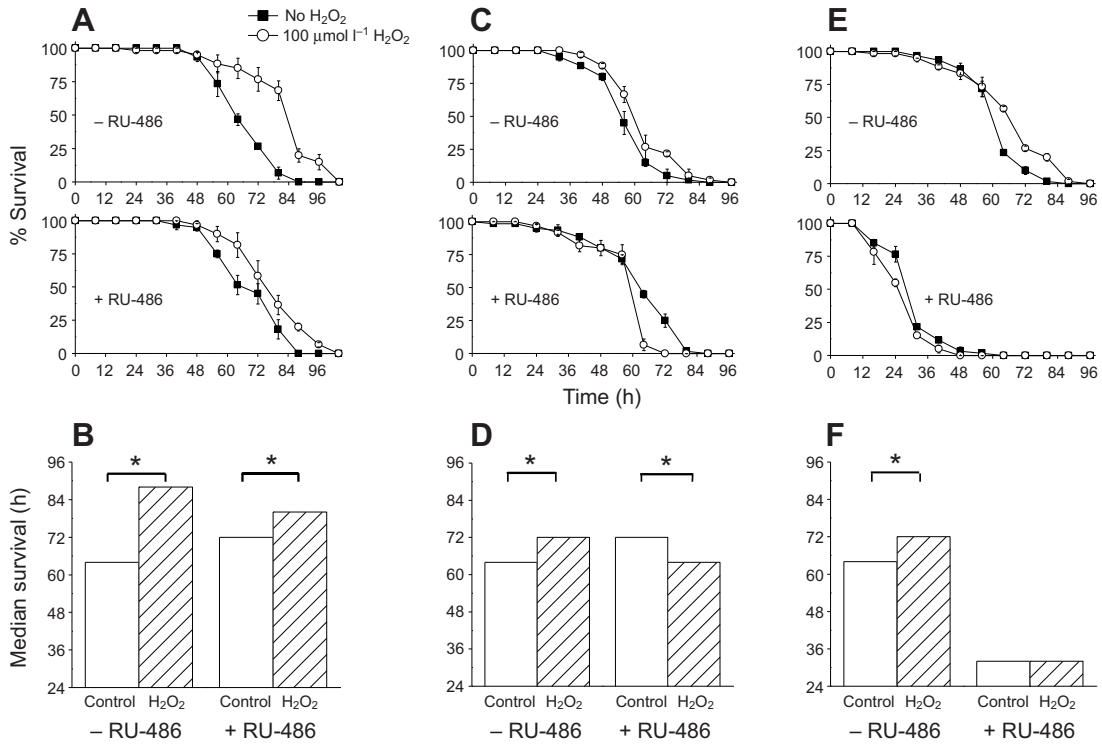

Fig. 6. Knockdown of *prosβ1* or *prosβ2* genes encoding proteasome subunits blocks the adaptive increase in oxidative stress tolerance of female progeny from (A,B) male w[1118] × female Act-GS-255B, (C,D) male *Prosβ2*^{RNAi} × female Act-GS-255B and (E,F) male *Prosβ1*^{RNAi} × female Act-GS-255B. In all cases flies were cultured with or without RU-486 for 6 days then pre-treated with 100 μmol l^{-1} H_2O_2 (±RU-486) for 24 h. After pre-treatment, flies were returned to vials with or without RU-486 for 16 h then challenged with 4.4 mol l^{-1} H_2O_2. Values are plotted as means ± s.e.m., where N=3. (A,C,E) Percentage survival. Values are plotted as means ± s.e.m., N=3. (B,D,F) Median survival time, N=60. Values marked with an asterisk indicate statistically significant differences (P<0.05) using a log-rank test.

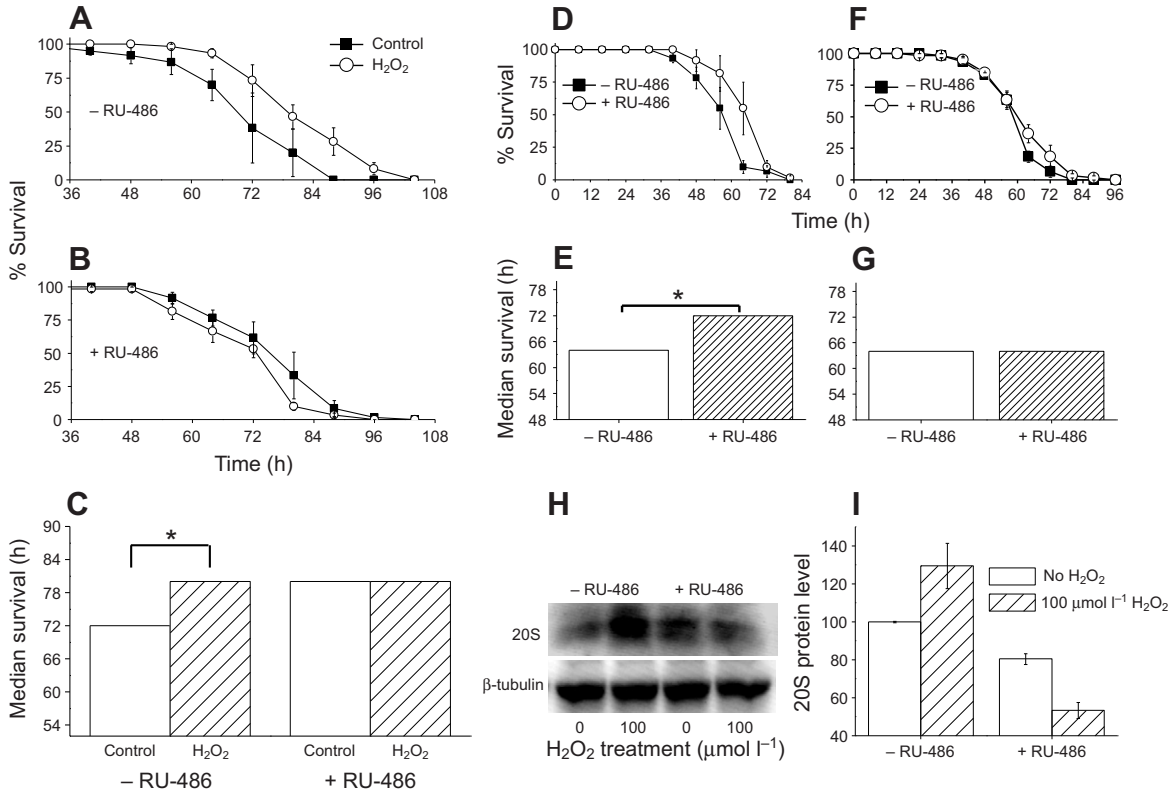

Fig. 7. (A,B) Knockdown of *cnc-C* blocks the adaptive increase in female fly oxidative stress tolerance. The female progeny of male *cnc-C*^{RNAi} × female Act-GS-255B were cultured without (A) or with (B) RU-486 for 3 days. After this, flies were pre-treated with 100 μmol l^{-1} H_2O_2 (±RU-486) for 24 h. Flies were then allowed to adapt without or with RU-486 for 24 h then challenged with 4.4 mol l^{-1} H_2O_2. Values are plotted as means ± s.e.m., *N*=3. (C) Median survival time for A and B, *N*=60. (D) Knockdown of *dkeap-1* increases oxidative stress tolerance. The female progeny of male *dkeap1*^{RNAi} × female Act-GS-255B were cultured with or without RU-486 for 5 days. Flies were then challenged with 4.4 mol l^{-1} H_2O_2. Values are plotted as means ± s.e.m., *N*=3. (E) Median survival time for C (*N*=60). (F) RU-486 treatment alone does not cause the increase in oxidative stress tolerance observed in D. The female progeny of male w[1118] × female Act-GS-255B were cultured with or without RU-486 for 5 days. Flies were then challenged with 4.4 mol l^{-1} H_2O_2. Values are plotted as means ± s.e.m., *N*=3. (G) Median survival time for F (*N*=60). (H) RNAi knockdown of *cnc-C* blocks the H_2O_2-induced increase in the 20S proteasome. The female progeny of male *cnc-C*^{RNAi} × female Act-GS-255B were cultured with or without RU-486 for 3 days. After this, flies were pre-treated with 100 μmol l^{-1} H_2O_2 (±RU-486) for 24 h. Flies were then allowed to adapt with or without RU-486 for 24 h and the results were analyzed by western blotting. (I) The gels run in H were run in triplicate and plotted as band density plots normalized to β-tubulin. Values are means ± s.e.m., *N*=3, and are shown as a percentage of controls (no RU-486, no H_2O_2). Values marked with an asterisk indicate statistically significant differences ($P \leq 0.05$) using a log-rank test.

progeny of these three crosses were then cultured with or without RU-486 for 7 days. RU-486 activates the Gene-Switch transcription factor, thereby inducing expression of the appropriate RNAi. One day prior to the end of the 7 day incubation, the female flies were transferred to vials containing Kimwipes soaked in 5% sucrose with or without 100 μmol l^{-1} H_2O_2 for 8 h, and then returned to vials containing RU-486 or ethanol for the remaining 16 h. At the start of the assay, the flies were transferred to vials containing 5% sucrose and a toxic dose of 4.4 mol l^{-1} H_2O_2. Fly survival was then scored every 8 h for the next 96 h. As a minor confounding issue, it was observed that RU-486 induced a small increase in tolerance to H_2O_2 challenge. This was controlled for by including controls with or without RU-486 in all experiments. In w[1118] × Act-GS-255B flies, an H_2O_2 pre-treatment-induced increase in tolerance to oxidative stress was observed, both with and without RU-486 treatment (Fig. 6A,B). The experiment was then repeated using the progeny from the two RNAi lines crossed with Act-GS-255B. In the *prosβ2* RNAi flies, it was found that flies not treated with RU-486 adapted to a similar extent to that seen for control female flies following H_2O_2 pre-treatment. In contrast, H_2O_2 pre-treatment in female flies that were also treated with RU-486 failed to increase tolerance to oxidative stress. In fact, H_2O_2 pre-treatment actually reduced oxidative stress tolerance in RU-486-treated females (Fig. 6C,D). Similarly, *prosβ1* RNAi flies not treated with RU-486 adapted to H_2O_2 pre-treatment to a similar extent to control flies. When flies were treated with both RU-486 and H_2O_2, however, not only did they lose the ability to adapt but also their tolerance to oxidative stress was severely reduced (Fig. 6E,F).

Role of CNC-C in oxidative stress adaptation in *D. melanogaster*

In both mammalian cells (Pickering et al., 2012) and *C. elegans* (Fig. 3), it was observed that Nrf2/SKN-1 is important for oxidative stress adaptation, and that it regulates the H_2O_2-induced increase in the 20S proteasome. We wished to test whether this was also the case in *D. melanogaster*. The *D. melanogaster* ortholog of Nrf2/SKN-1 is CNC-C, which has also been shown to be an important regulator of oxidative stress tolerance (Sykiotis and Bohmann, 2008; Sykiotis and Bohmann, 2010). First, to test whether CNC-C is involved in adaptation, a line expressing RNAi against *cnc-C* (Sykiotis and Bohmann, 2008; Sykiotis and Bohmann, 2010) was crossed with Act-GS-255B, and adaption experiments were performed on the progeny as for the proteasome RNAi lines. As before, an increase in oxidative stress tolerance was observed

with H_2O_2 treatment in flies not treated with RU-486 (Fig. 7A), while no adaptive increase in oxidative stress tolerance was observed in RU-486-treated flies (Fig. 7B). In fact, H_2O_2 treatment caused a reduction in oxidative stress tolerance (Fig. 7A–C) in RU-486-treated female flies.

DKEAP-1 is a repressor of CNC-C and has a similar function to that of Keap-1, which functions as a repressor of Nrf2 in mammalian cells (Sykiotis and Bohmann, 2008). As before, the *dkeap-1* RNAi line was crossed with Act-GS-255B and the progeny were then treated with RU-486 for 5 days. The flies were subsequently challenged with a toxic dose of H_2O_2 and survival was measured as before. It was found that knock-down of *dkeap-1* caused a significant increase in resistance to oxidative stress (Fig. 7D–G). We were next interested to determine whether the induction of the 20S proteasome is dependent on CNC-C/Nrf2. To test this, *cnc-C*^{RNAi} × ACT-GS-255B flies were prepared and pre-treated as in Fig. 7A. The flies were then lysed and the proteins analyzed by western blotting with antibodies directed against α-subunits of the 20S proteasome, or β-tubulin as a loading control. It was observed that H_2O_2 treatment produced an increase in the expression of the 20S proteasome subunit that was suppressed in flies treated with RU-486. These results are supportive of a role for *cnc-C* in regulating the H_2O_2-induced increase in the 20S proteasome (Fig. 7H,I).

DISCUSSION

In mammalian cells, it has been shown that H_2O_2-induced adaptation to oxidative stress occurs through a pathway that is strongly dependent on an Nrf2-mediated increase in the 20S proteasome (Pickering et al., 2010; Pickering et al., 2012). The model organisms *C. elegans* and *D. melanogaster* have also been shown to be capable of adaptation to oxidative stresses through mild exposure to heat shock, 100% oxygen or the redox quinone juglone in worms, and heat shock or irradiation in flies (Cypser and Johnson, 2002; Lithgow et al., 1995; Moskalev et al., 2009; Przybysz et al., 2009). The present results demonstrate that both model organisms are capable of such adaptation with H_2O_2 pre-treatment. As in mammalian cells (Pickering et al., 2010), it was found that this adaptive response involves an increase in proteolytic activity and increased expression of the 20S proteasome, but not of the 26S proteasome. Moreover, it was found that the increase in 20S proteasome expression in both worms and flies, as in mammalian cells, is important for the adaptive response, and that this increase is mediated by the SKN-1 and CNC-C orthologs of the mammalian Nrf2 transcription factor, respectively. These studies indicate conservation of the adaptive response to oxidative stress across a wide biological diversity, and demonstrate that stress mechanisms operative in cell culture also apply in disparate intact organisms.

Stress adaption, or conditioning hormesis, has been widely reported in *C. elegans*, but it is typically studied in terms of changes in lifespan, with only a few examples of changes in oxidative stress tolerance (Cypser and Johnson, 2002; Lithgow et al., 1995). It was shown in previous reports that exposure to high (10 mmol l^{-1}) concentrations of H_2O_2 induces SKN-1 and causes its translocation to the nucleus (An and Blackwell, 2003; An et al., 2005; Oliveira et al., 2009). It appears that much lower (1 μmol l^{-1}) adaptive concentrations of H_2O_2 also induce SKN-1 and cause its translocation to the nuclei in cells throughout the intestine of *C. elegans*. In addition, using *skn-1* RNAi and *skn-1* mutants, it was shown that this induction and translocation is important for the adaptive increases in proteasome expression and oxidative stress resistance.

Oxidant- or radiation-induced adaptation to oxidative stress in *D. melanogaster* has previously been reported (Moskalev et al., 2009). Here, it was confirmed that upon H_2O_2 pre-treatment flies become more resistant to oxidative stress and their cellular proteolytic capacity increases. This increase in proteolytic capacity was shown to be largely dependent on the 20S proteasome, which is consistent with mammalian cell culture studies (Pickering et al., 2010). Finally, these data indicate that CNC-C, the *Drosophila* ortholog of mammalian Nrf2 and *C. elegans* SKN-1, mediates adaptive increases in stress resistance, at least in part through induction of 20S proteasome expression.

An important aspect of this study is the use of short-term RNAi treatments as a means of blocking new protein synthesis without significantly altering the baseline expression level of the target protein. This was achieved in *C. elegans* by feeding worms RNAi for 24 h and in flies by feeding with RU-486 for 5–7 days in adulthood. One concern in the use of RNAi to deplete a protein from an animal is that this approach may cause additional unwanted physiological changes such as either inhibition of normal cell function or production of a stress response. Here, such unwanted physiological changes were reduced or avoided by employing short-term RNAi treatments.

One long-term goal for the present work was to develop viable animal models for aging, consisting of short-lived organisms that recapitulate major stress-adaptive mechanisms that operate in mammals. The present data demonstrate that *C. elegans* and *D. melanogaster*, both of which have long been among the most important organisms for aging models, share with mammalian cells a capacity for oxidative stress adaptation that relies on increased 20S proteasome synthesis, mediated by well-conserved (Nrf2/SKN-1/CNC-C) signal transduction pathways.

LIST OF ABBREVIATIONS

AMC	7-amino-4-methylcoumarin
CNC-C	*D. melanogaster* cap'n'collar transcription factor (an ortholog of mammalian Nrf2)
H_2O_2	hydrogen peroxide
Nrf2	nuclear factor (erythroid-derived 2)-like 2
PA28αβ	proteasome activator 28αβ
PAS-5	proteasome α-subunit 5
PAS-7	proteasome α-subunit 7
PBS-3	Proteasome β-subunit 3
PBS-5	Proteasome β-subunit 5
PBS-6	proteasome β-subunit 6
RPN-10	proteasome regulatory particle non-ATPase-like 10
SKN-1	*C. elegans* SKiNhead-1 transcription factor (an ortholog of mammalian Nrf2)
Suc-LLVY-AMC	succinylated peptide *N*-succinyl-leucine-leucine-valine-tyrosine-7-amino-4-methylcoumarin (used as a peptide substrate to measure proteolytic capacity)

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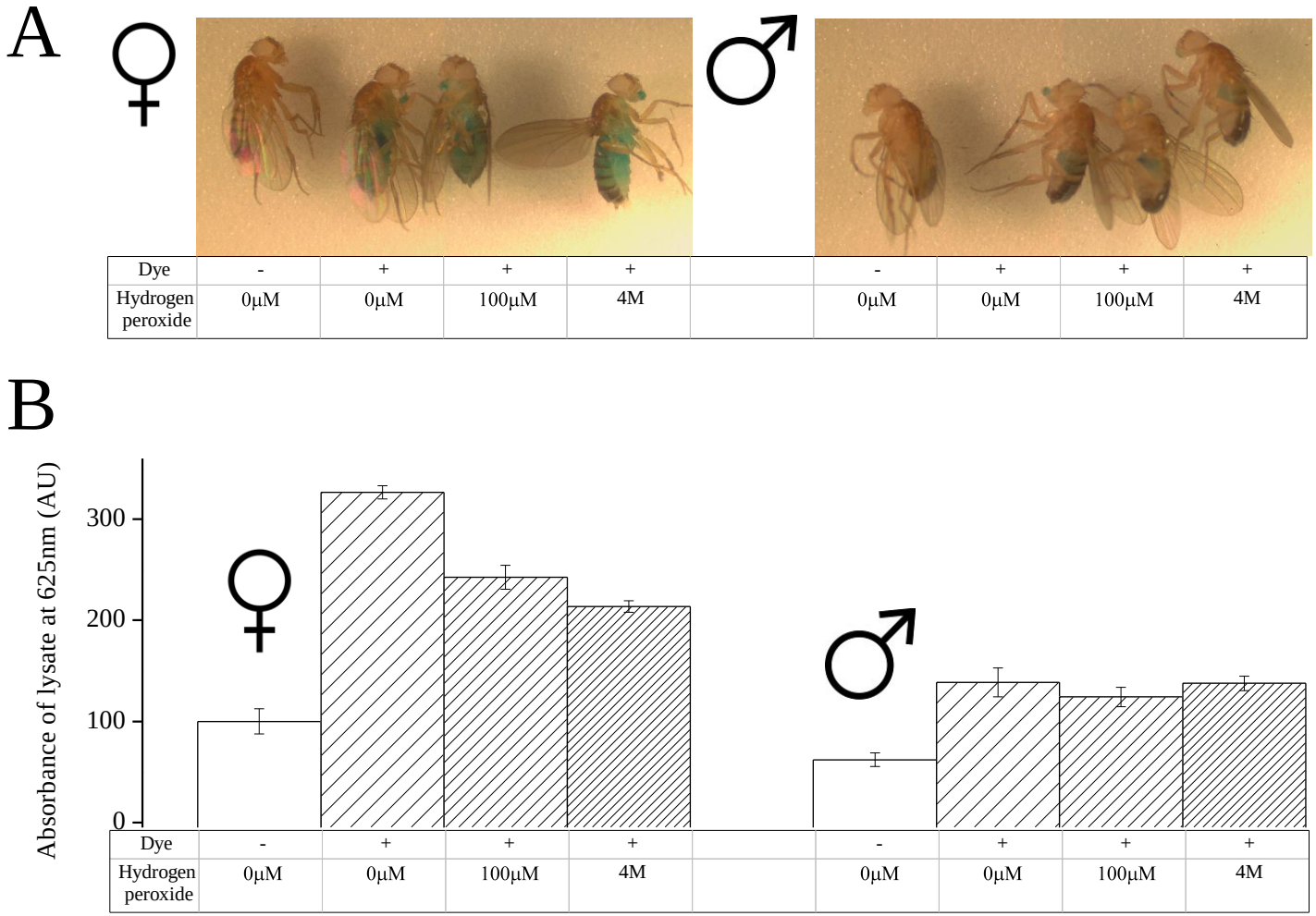

Fig. S1: Uptake of sucrose/H_2O_2 solution by *Drosophila melanogaster*. **A.** Female and male Drosophila ingest a solution of sucrose, and various doses of H_2O_2 over the 8 h H_2O_2 pretreatment, though females ingest significantly more. In this background experiment w[1118] flies were placed on a ½ Kim-wipe© soaked in a solution of 5% sucrose + 0μM, 100μM or 4.4M H_2O_2 + 10% blue food color (Amerifoods, Los Angeles, CA, USA) for 8 h. Flies were then visualized under a stereoscope. Visible dye staining is observed around the gut and mouth of all flies treated with the food dye. **B.** Five flies from each of the treatment conditions in **A** were suspended in 300μl of DI water then lysed using an electronic pestle. After this, the samples was centrifuged to removed un-lysed particles. The supernatants were studied in triplicate on 96 well plates and absorbance at a wavelength of 625nm was measured.

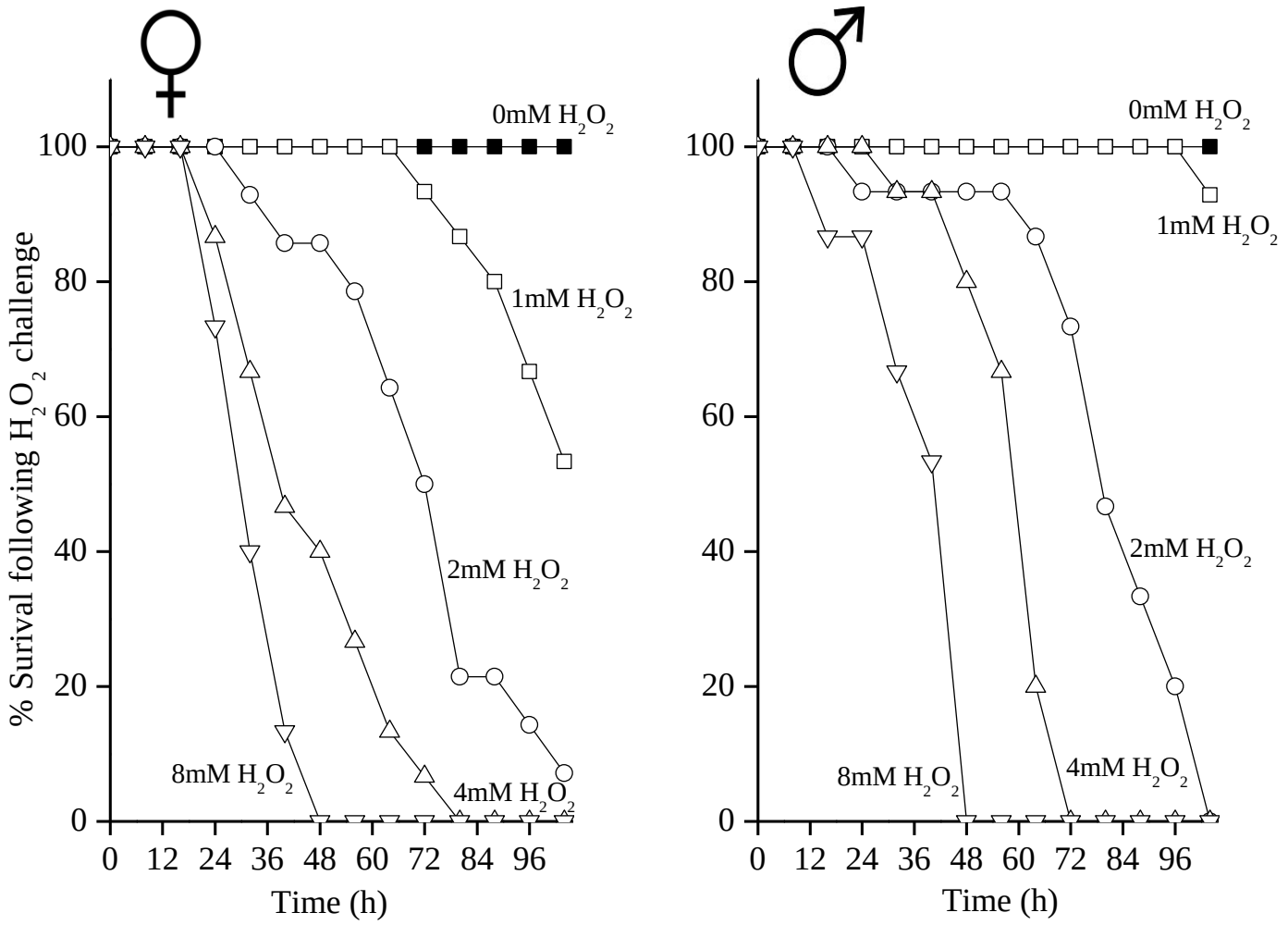

Fig. S2: Survival of *Drosophila melanogaster* following H_2O_2 Challenge. H_2O_2 challenge causes a decline in Drosophila survival which is independent of incubation on 5% sucrose. Samples of 15 female or male w[1118] flies were placed on a ½ Kim-wipe© soaked in a solution of 5% sucrose + 0M, 1M, 2M, 4M or 8M H_2O_2. Survival was then scored every 8 h over a total period of 104 h, which was the length of the longest survival assay run in the paper.