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SEXUAL DIMORPHISM IN OXIDANT-INDUCED ADAPTIVE HOMEOSTASIS IN MULTIPLE WILD-TYPE *D. MELANOGASTER* STRAINS

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Abstract

Sexual dimorphism includes the physical and reproductive differences between the sexes, including differences that are conserved across species, ranging from the common fruit fly, *Drosophila melanogaster*, to humans. Sex-dependent variations in adaptive homeostasis, and adaptive stress responses may offer insight into the underlying mechanisms for male and female survival differences and into differences in chronic disease incidence and severity in humans. Earlier work showed sex-specific differences in adaptive responses to oxidative stressors in hybrid laboratory strains of *D. melanogaster*. The present study explored whether this phenomenon is also observed in wild-type *D. melanogaster* strains Oregon-R (Or-R) and Canton-S (Ca-S), as well as the common mutant reference strain w[1118], in order to better understand whether such findings are descriptive of *D. melanogaster* in general. Flies of each strain were pretreated with non-damaging, adaptive concentrations of hydrogen peroxide (H₂O₂) or of different redox cycling agents (paraquat, DMNQ, or menadione). Adaptive homeostasis, and changes in the expression of the proteasome and overall cellular proteasomal proteolytic capacity were assessed. Redox cycling agents exhibited a male-specific adaptive response, whereas H₂O₂ exposure provoked female-specific adaptation. These findings demonstrate that different oxidants can elicit sexually dimorphic adaptive homeostatic responses in multiple fly strains. These results (and those contained in a parallel study [1]) highlight the need to address sex as a biological variable in both fundamental science, clinical research, and toxicology.

Keywords

proteasome; proteostasis; adaptive homeostasis; oxidative stress; hydrogen peroxide; redox cycling

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CONTRIBUTIONS
LCDP, SW, JT, and KJAD conceived and designed the studies; LCDP and SW performed the experiments; LCDP, SW, JT, and KJAD analyzed the data; LCDP, SW, JT, and KJAD interpreted the results; LCDP, SW, JT, and KJAD drafted the manuscript; LCDP, SW, JT, and KJAD edited and revised the manuscript. All authors approved the final version of the manuscript. Experimental procedures were performed at the University of Southern California, USA.

INTRODUCTION

Across several species, females consistently outlive males [2–7]. In humans the robustness of this trend begins as early as childhood (birth to age 5), wherein females uniformly show greater survival. This trend is continued into later life (age 50), with women comprising 90% of supercentenarians [8]. Women have a lower mortality risk from certain chronic diseases [9], and the majority of current longevity interventions favor females [10]. Some findings point to genetic factors [11–14], hormonal exposure [15, 16], and mating [17, 18] as the major causes for sex differences [19]. Nor are cellular differences between the sexes entirely attributable to hormonal exposure [20], as genetic differences result in varying embryonic growth rates [21, 22], and higher sensitivity in male hippocampal neuronal cells following peroxynitrite exposure compared to female neuronal cells [23]. Additionally, hormonal ablation does not completely eliminate sex-specific responses in tumor progression [24], or differences in sensitivity to carcinogens [25], nor in survival differences in pediatric stroke studies [26], suggesting that additional mechanisms may be involved.

Key signaling pathways, such as the nutrient sensing, insulin/insulin-like growth factor (IGF-1) pathway, show a female-specific benefit. Female mice heterozygous for IGF-1 mutation survive longer and are more stress-resistant compared to their wild-type counterparts, whereas heterozygous males show no difference [27, 28]. Tissue-specific differences also arise in response to changes in nutrient signaling, as short-term caloric restriction triggers increased oxidative phosphorylation to a greater extent in females than in males [29]. Similarly, male *D. melanogaster* show reduced heat tolerance following subsequent removal of the insulin-like receptor homolog, InR [30], whereas females exhibit increased lifespan. The impact of the insulin-sensing pathway also appears to be sex-specific in humans, as females show greater sensitivity to insulin, as evidenced by differences in glucose utilization in both muscle and liver tissues [31].

Sex-bias in the cellular response to acute oxidative stress has been reported for humans and mice, where cells from females generally show greater stress resistance than do cells from males [32]. Furthermore, sex-dependent difference are also evident in adaptation, or adaptive homeostasis [33], albeit less-explored. The adaptive homeostatic response is a signaling cascade induced in cells or organisms by a non-toxic amount of an oxidant or compound that protects against future, more damaging, insults. Early cell-culture studies demonstrated the adaptive increase in multiple stress responsive enzymes, including the 20S proteasome [34–36] and the Lon protease [37, 38] in response to adaptive doses of H₂O₂. Replication of the adaptive methodology in model organisms, most notably *D. melanogaster* [39–41] and *C. elegans* [42, 43] demonstrated that adaptation to H₂O₂ is also observed in these multicellular animals.

The model organism, *D. melanogaster* is an excellent system in which to study sex differences in life span regulation, due to the short life span and the presence of both sexes. Comparisons between males and females for several *Drosophila* genotypes [17, 44, 45], indicates that females, on average, are longer-lived than their male counterparts. Unique physiological characteristics of females, including larger body size [46] and greater rate of

regeneration of the gut epithelium [47], are interesting and testable possible explanations for differences in the stress response between the sexes, including differences in the adaptive stress response.

The purpose of the present study was to assess the sex-specific differences in the adaptive stress response in wild-type and w[1118] *D. melanogaster* strains. The sex-dependent response of the superoxide-producing redox cycling agent, paraquat was compared to the short-term oxidant, hydrogen peroxide. 3 day old flies of the three strains: Canton-S (Ca-S), Oregon-R (Or-R), and w[1118] were pretreated with micromolar, 'adaptive' amounts of each oxidant. Following pretreatment, flies were subjected to semi-lethal concentrations of either paraquat or hydrogen peroxide to measure changes in survival. The 20S proteasome expression and activity were measured following pretreatment. Additionally, other redox cycling agents, 2,3-Dimethoxy-1,4-naphthoquinone (DMNQ), and menadione, were tested to measure sex-specific adaptive differences. These experiments (and those contained in an additional study [1]) reveal that the redox-cyclers consistently trigger a male-specific adaptive response, whereas H₂O₂ caused a female-specific adaptive response. Overall this work improves our understanding of the sex-specific differences in adaptive responses to oxidative stress. More importantly, it emphasizes the need to address sex as a vital biological variable in aging, and in determining disease susceptibility and severity.

MATERIALS AND METHODS

D. melanogaster culture

Three common strains of *D. melanogaster* were utilized: Canton-S, Oregon-R, and w[1118]. These strains were selected due to their high usage as standard control strains in *D. melanogaster* genetics, with each strain having been utilized in at least 2000 publications (PubMed). These strains have also been extensively utilized in lifespan and stress-resistance studies and provide the genetic background for genetically modified strains. For further explanation of these standard strains, please see the following [1]. All strains were cultured at 25°C, with 12 hour light/dark cycles, on a standard agar/dextrose/corn meal/yeast media [48]. Flies were collected over 48 hours from pre-cleared bottles prior to treatments.

D. melanogaster challenge assay

Ten flies were transferred into vials with a Kimwipe soaked in 1mL of 5% sucrose for 24 hours. Upon treatment initiation, flies were transferred to vials with 1mL of 5% sucrose and increasing concentrations of H₂O₂ [0M-8M] or paraquat [0mM-40mM] as indicated. Survival was scored every 8 hours, until all flies were dead.

D. melanogaster adaptation

24 hours prior to treatment, 10 flies were transferred to vials containing 1mL of 5% sucrose. Upon treatment, flies were transferred to vial with 1mL of 5% sucrose and low amounts of H₂O₂ [0μM-100μM] or paraquat [0μM-10μM], as indicated, for 8 hours, and subsequently transferred to vials containing only 1mL of 5% sucrose for an additional 16 hours. Afterwards, flies were transferred to vials containing a toxic dose of H₂O₂ [4.4M] or paraquat [30mM]. Survival was scored every 8 hours, until all flies were dead.

Pretreatment with various oxidants

Ten flies were transferred to vials containing 1mL of 5% sucrose. Upon treatment initiation, flies were transferred to vials containing menadione [0μM-1mM], DMNQ [0μM-10μM], paraquat [0μM-10μM] or H₂O₂ [0μM-100μM] for 8 hours before being placed back onto vials with only 1mL of 5% sucrose for an additional 16 hours. Afterwards flies were frozen for down-stream processing.

Preparation of *D. melanogaster*

Flies were homogenized in 200μL proteolysis buffer (50mM Tris/HCl, 20mM KCl, 5mM MgAc, 1mM DTT, pH 7.5) using an electric pestle. Further lysis was conducted by three 'freeze-thaw' cycles, consisting of 5-minute intervals on dry ice, followed by incubation in water, and vortexed. Samples were centrifuged for 10,000g for 10min at 4°C to remove cuticle fragments. Protein concentration was measured using the Bicinchoninic acid assay (BCA) reducing agent compatible kit (no. 23252, ThermoFisher Scientific).

Fluoropeptide Proteolytic Activity Assays

5μg of whole fly lysate was aliquoted, in triplicate, to 96-well plates. Proteasome's chymotrypsin-like activity was measured by adding 2μM of the proteasomal β5-specific fluorogenic substrate, Suc-LLVY-AMC (no. 539141, Calbiochem). Lysate was incubated at 37°C, and fluorescence readings were recorded every 10 minutes for 4h using an excitation/emission of 355nm/444nm. Fluorescence units were converted to moles of free 7-amino-4-methylcoumarin (AMC), using an AMC standard curve (no. 164545, Merck), with background subtracted. To measure proteolytic inhibition, 20μM of the proteasome inhibitor, lactacystin (no. 80052–806, VWR) was added directly to lysate, and incubated on plate shaker for 30min at 300rpm, after which, substrate was added.

Western Blots

10μg of whole fly lysate was run on a 4–15% gradient SDS-PAGE gel (no. 4568084, Bio-Rad) for 1 hour at 100V before being transferred at 4°C to a PVDF membrane (no. 1620177XTU, Bio-Rad). The goat polyclonal anti-Actin-HRP antibody, conjugated to horseradish peroxidase (1:1000 dilution, no sc-1616, Santa Cruz Biotechnology) was used as the protein loading control. Monoclonal antibody against the α-subunit of the 20S core proteasome of *D. melanogaster* was used (1:100 dilution, no. sc-65755, Santa Cruz Biotechnology).

Immunoprecipitation (IP)

200 flies were homogenized in 400μL proteolysis buffer (50mM Tris/HCl, 20mM KCl, 5mM MgAc, 1mM DTT, pH 7.5) using an electric pestle. Further lysis was conducted by three 'freeze-thaw' cycles, consisting of 5-minute intervals on dry ice, followed by incubation in water, and vortexed. Samples were centrifuged for 10,000g for 10min at 4°C to remove cuticle fragments. Protein concentration was measured using the Bicinchoninic acid assay (BCA) reducing agent compatible kit (no. 23252, ThermoFisher Scientific). Each IP sample was normalized to have 400μg protein in a final volume of 300μL proteolysis buffer. Next, 20μL of washed protein G Sepharose 4B beads (no. 10–1242, ThermoFisher

Scientific) were added to each sample and placed on an end-over-end shaker for 30min at 4°C to pre-clear the lysate. (Antibody binding beads were washed twice by first adding 500µL 1X PBS, inverting the sample twice to mix, and centrifuging at 2000rpm for 1min at 4°C to pellet the beads, at which point the supernatant was removed). Afterwards, samples were centrifuged at 2000rpm for 1min at 4°C to pellet the beads and the lysate was transferred to fresh tubes, at which point 20µL of the monoclonal antibody against the α-subunit of the 20S core proteasome of *D. melanogaster* (no. sc-65755, Santa Cruz Biotechnology) was added and samples were rotated at 4°C, overnight. Next, 40µL of washed antibody binding beads were added to each IP and rotated at 4°C, overnight. Samples were centrifuged at 2000 rpm for 2min at 4°C. Afterwards, the 'Flow-through' (supernatant) was transferred to fresh tubes for down-stream analysis. Beads were washed once with 1x mPER buffer (no. 78501, ThermoFisher Scientific) and three times with ice-cold 1x PBS solution. Samples were rotated for 5min between washes. Beads were eluted by adding 50µL of sample buffer containing 5% SDS and heated at 95°C for 5min prior to loading on an 10% SDS-PAGE gel.

RESULTS

Sex differences in resistance to hydrogen peroxide toxicity

In a previous study, sex-specific differences were identified in the adaptation to hydrogen peroxide (H₂O₂) in several laboratory strains of flies [39–41, 43]. To determine if the sex-specific differences in H₂O₂ adaptation are observed in wild-type *D. melanogaster* strains, two common wild-type strains were analyzed, Canton-S (Ca-S) and Oregon-R (Or-R), along with the common reference strain w[1118]. Three-day old progeny from each strain were fed various concentrations of H₂O₂ [0M–8M], and survival was scored every 8 hours. In all three strains, males showed similar sensitivity to challenge doses greater than 2M (Figure 1A,C,E), with males of the w[1118] strain showing the greatest resistance at 1M, matching earlier findings [43]. Females showed similar sensitivity to increasing concentrations of H₂O₂, with greatest resistance observed for w[1118] (Figure 1B,D,F).

Hydrogen peroxide pretreatment results in female-specific adaptation

Adaptation, or adaptive homeostasis [33], occurs for a broad spectrum of oxidative (and many other) stressors [33, 49, 50]. Exposure to adaptive doses (micromolar amounts) of H₂O₂ have been previously shown to upregulate cytoprotective genes, which protect against future, potentially more damaging, oxidative insults. Prior studies in cell culture [34, 36], and the model organisms *C. elegans* [42, 43] and *Drosophila melanogaster* [39, 40, 43] have shown that H₂O₂ pretreatment increases survival from a subsequent toxic H₂O₂ insult, and in *D. melanogaster* this H₂O₂ adaptation was shown to be female-specific [39, 40, 43]

Here, we sought to determine if H₂O₂ pretreatment in common laboratory strains also caused a female-specific adaptive response [39, 40, 43]. To do so, flies of the three strains were collected and placed, 24 hours prior to pretreatment, upon 5% sucrose. Afterwards, flies received either no H₂O₂ or adaptive concentrations of H₂O₂ [10µM or 100µM] for 8 hours, before being placed back onto 5% sucrose for an additional 16-hour recovery. Flies were challenged with 4.4M H₂O₂ and survival was scored every 8 hours. In all three strains,

males, irrespective of pretreatment, showed no difference in survival (Figure 2A–C). The lack of adaptation in males was not due to insufficient pretreatment concentrations, as higher doses were detrimental to survival [43]. In contrast, females of all three strains showed longer survival times compared to females that did not receive the pretreatment (Figure 2D–F). Pretreatment with adaptive concentrations of H_2O_2 [10 μ M, 100 μ M, or 1000 μ M] also resulted in females (Figure 2G,I,K), but not males (Figure 2H,J,L), exhibiting higher levels of the 20S proteasome, as measured by 20S proteasome α subunit protein levels.

Hydrogen peroxide adaptation involves a female-specific increase in proteasomal proteolytic capacity

Next, we measured proteasome function to determine if there was an adaptive change in overall proteolytic capacity. Progeny were subjected to adaptive doses of H_2O_2 [0 μ M–1000 μ M] for 8 hours before a 16-hour recovery and subsequent collection. Proteasome function was assessed in whole fly lysates by the degradation of the fluorogenic substrate Suc-LLVY-AMC. Similar to the inability to adapt in Fig 2, males showed no change in proteolytic activity upon H_2O_2 pretreatment (Figure 3A,C,F). In contrast, pretreated females showed higher amounts of proteolytic capacity (Figure 3B,D,E). To confirm that the higher levels of proteolytic activity was due to the proteasome, the proteasome inhibitor Lactacystin was added to the lysate from pretreated females and, as expected, proteolytic activity decreased (see Figure 1A in [1]). Lysate from pretreated males also showed decreased basal activity upon addition of the proteasome inhibitor (see Figure 1B in [1]).

Sex differences in resistance to paraquat toxicity

The redox cycling agent, paraquat (methyl viologen dichloride) accepts electrons from intracellular one-electron donors (such as mitochondrial complexes I and III and cytochrome P450 reductase) to generate the paraquat radical, and then donates those single electrons to molecular O_2 resulting in superoxide ($O_2^{\bullet-}$) generation [51]. Since this process regenerates oxidized paraquat, it can be continuously repeated to generate a constant stream of $O_2^{\bullet-}$ in a process called redox cycling. Paraquat (PQ) has been used extensively as an oxidant in *D. melanogaster* studies, and resistance to paraquat often correlates with strain life-span [52]. To determine if paraquat toxicity is similar among the three *D. melanogaster* strains, 3-day old progeny were subjected to increasing concentrations of paraquat [0mM–40mM] to assess survival. Males of the Ca-S strain showed greater sensitivity to paraquat toxicity compared to males of the Or-R or w[1118] strains (Figure 4A,C,E). Similarly, females of the Ca-S strain showed the greatest sensitivity to paraquat toxicity, whereas Or-R and w[1118] females had greater resistance at the same paraquat concentrations (Figure 4B,D,F).

Paraquat pretreatment results in male-specific adaptation

Next, the potential ability to adapt following adaptive exposure to paraquat was tested. We have recently reported that males, but not females adapt to paraquat pretreatment using limited studies of common hybrid laboratory strains [39]. To determine whether adaptation is observed in males from wild-type and w[1118] strains, progeny were either placed on 5% sucrose or were pretreated with adaptive doses of paraquat [1 μ M or 10 μ M] before being subjected to a challenge dose of paraquat [30mM]. Pretreated males of all three strains

showed longer survival times compared to those that did not receive the pretreatment (Figure 5A–C, see Table 2 [1]). In contrast, females of all three strains, regardless of pretreatment, showed no change in survival (Figure 5D–F, see Table 2 [1]).

To determine if the adaptive increase in survival was associated with an increase in proteasome expression, western blots were performed to measure proteasome levels. Upon paraquat pretreatment, females showed no change in proteasome levels (Figure 5G,I,K). In contrast, males showed higher protein levels of the proteasome (Figure 5H,J,L).

Paraquat, adaptation involves a male-specific increase in proteasomal proteolytic capacity

Because paraquat pretreatment resulted in increased expression of the proteasome and of survival in males, we sought to determine if proteasome capacity was also increased. Progeny were pretreated with either no paraquat or low adaptive doses [$1\mu\text{M}$ – $100\mu\text{M}$] for 8 hours before an additional 16-hour recovery prior to collection. Whole fly lysate was assessed for proteasome activity by the addition of the fluorogenic peptide, Suc-LLVY-AMC. Males of each strain showed higher amounts of proteolytic capacity compared to controls (Figure 6A,C,E). In contrast, no female flies of any strain exhibited higher proteasomal proteolytic capacity following paraquat treatment; in fact, Or-R females showed a significant decrease at the highest paraquat dose (Fig. 6B,D,F). Importantly, the higher levels of proteolysis in pre-treated males was shown to be dependent upon the proteasome. Lysates from males and females either pre-treated or not with paraquat were also incubated with the proteasome inhibitor, lactacystin. No change in proteolytic capacity was measured in paraquat pre-treated females incubated with lactacystin (see Figure 1C in [1]), but lactacystin inhibition blocked the adaptive rise in paraquat pretreated males (see Figure 1D in [1]).

Pretreatment with multiple redox-cyclers results in a male-specific rise in proteolysis

Because paraquat showed a male-specific proteasome increase (in contrast with H_2O_2), we sought to address whether this phenomenon is a feature of redox cycling agents in general. To test this hypothesis, 2,3-Dimethoxy-1,4-naphthoquinone (DMNQ) and menadione (an analog of 1,4 naphthoquinone with vitamin K activity) were studied. DMNQ is a redox cyclers, and during its one electron reduction, and subsequent oxidation by molecular O_2 , acts as a continual generator of superoxide [53]. DMNQ was tested to determine if it would cause an adaptive rise in proteasomal proteolytic capacity and if the response would be male-specific. Progeny from the three strains were pretreated with increasing concentrations of DMNQ [500nM – $10\mu\text{M}$]. Following pretreatment, whole fly lysate was assayed for proteolytic capacity of the proteasome following the addition of the fluorogenic peptide, Suc-LLVY-AMC. Upon pretreatment, males of all three strains showed higher proteolysis levels, measured by increased fluorescence (Figure 7A,C,E). Strikingly, females pretreated with DMNQ, showed no change in proteolytic capacity (Figure 7B,D,F). Together, suggesting DMNQ may play a similar role in triggering a male-specific response, as previously shown with paraquat (see above) [39].

Next, menadione, commonly used as a nutritional supplement due to its Vitamin K activity, was explored. Menadione acts as both a redox cyclers and arylating quinone, and is reported

to increase the intracellular concentration of superoxide [54]. Earlier studies found pretreatment with a low concentration of menadione was able to protect *S. cerevisiae* from future insults [55], suggesting menadione may act as an inducer of an adaptive stress response. Here, low concentrations of menadione [500nM-1mM] were fed to progeny of the three *D. melanogaster* strains. Following pretreatment, whole fly lysate was assayed for changes in the proteasome proteolytic capacity. Males pretreated with menadione exhibited an elevated capacity to degrade the small fluorogenic peptide Suc-LLVY-AMC (Figure 8A,C,E), whereas females pretreated with menadione showed no change in proteolysis (Figure 8B,D,F). Thus several redox cycling agents are capable of inducing not only the stress response, but also a male-specific adaptive response.

The adaptive proteolytic response is dependent upon the proteasome

Finally, we sought to determine if the adaptive increase in proteolysis was primarily dependent upon the proteasome. To address this question, flies of the Ca-S strain were pretreated with or without an adaptive dose of H₂O₂. Following pretreatment, fly lysates were immunoprecipitated using the *D. melanogaster* specific monoclonal antibody against the 20S proteasome α subunit, to remove the proteasome from the lysate. Following removal of proteasomes from the flow-through, proteolytic activity in the flow-through and input lysate was assessed. Additionally, efficiency of proteasome removal was confirmed by western blot (see Figure 2 in [1]). The fluorogenic peptide, Suc-LLVY-AMC was used to assay changes in proteasome activity, with increased fluorescence indicative of higher proteasome degradation capacity. Higher proteolytic activity was evident in the input lysate from pretreated females (Figure 9A). Lysate from the female flow-through showed no change in proteolytic activity, showing that the higher proteolytic activity is primarily dependent upon the proteasome. In males, although the input lysate showed no adaptive change in proteolytic activity following H₂O₂ pretreatment, there was a significant decrease in the basal proteasome activity in the flow-through lysate (Figure 9B).

DISCUSSION

Redox cycling agents and hydrogen peroxide are extensively used as oxidizing agents in redox biology and free radical research. Yet, only recently has the relationship between sex-specific adaptive responses and the type of oxidant employed begun to be explored [39]. Our previous studies with laboratory strains of flies showed that females, but not males adapt to H₂O₂ stress [39–41, 43] and that males, but not females, adapt to paraquat stress [39]. The present study demonstrates that additional redox cycling agents (DMNQ and menadione) induce a male-favored adaptive response similar to paraquat, whereas females show adaptation only upon hydrogen peroxide pretreatment. These results demonstrate the sex-specific adaptive response is observed in wild-type *D. melanogaster* strains, including the Oregon-R strain and the relatively short-lived, highly oxidant-sensitive Canton-S (Ca-S) strain [56, 57], along with the common w[1118] reference strain. These results further establish *Drosophila melanogaster* as a tractable invertebrate model system to study sex-specific adaptive responses, which may help elucidate the underlying mechanistic sex differences evident in higher organisms.

Adaptation, or 'adaptive homeostasis' is a highly conserved process, wherein cells, tissues, and whole organisms transiently activate various signaling pathways in response to short-term mild perturbations, triggering transcriptional and translational modifications. These adjustments protect against future, potentially more-damaging insults [33]. We feel it is important to differentiate between the physiological concept of adaptive homeostasis [33] which posits that discrete signal transduction pathways (such as Nrf2) mediate transient adaptation, and the more toxicological concept of hormesis [33, 49, 50] in which actual damage or toxicity itself is proposed to be the mechanistic 'signal' for adaptation. We have begun to address this question in previous publications. Thus, in studies using mouse embryonic fibroblasts (MEF) [32], we have shown that low concentrations of H₂O₂ (e.g. 10.0μM) which cause no cellular damage whatever, produce increases in Nrf2 levels and increased Nrf2 translocation to the nucleus, along with induction of increased Proteasome and Lon protease synthesis and increased stress resistance (after a suitable period of adaptation). In contrast, higher (but non-lethal) concentrations of H₂O₂ (e.g. 100.0μM) cause discernable cellular damage and slow growth, but still increase Nrf2 levels, Nrf2 nuclear translocation, and increased Proteasome and Lon protease synthesis albeit at lower levels, and still increase stress resistance but to a lesser extent than do low H₂O₂ concentrations. Similar results were obtained with low (non-damaging) *versus* higher (mildly damaging) levels of peroxyxynitrite, paraquat, and menadione. Importantly, all adaptive responses were transient, and all were blocked by Nrf2 inhibitors and/or knock-down. From these studies one may conclude that cells in culture use the Nrf2 signal transduction pathway to adapt very well to low signaling levels of agents such as H₂O₂, peroxyxynitrite, paraquat, and menadione. In contrast, cultured cells are less able to undergo adaptive homeostasis when damaging levels of the same agents are employed.

The above studies provide important basic information for cells in culture, but do not tell us if a whole organism or animal would respond similarly. Therefore, we initiated studies in *C. elegans* worms and *D. melanogaster* fruit flies. Our results [37, 38] all reveal a consistent trend; that low, non-damaging, or signaling levels of various oxidants and redox cycling agents effectively induce transient adaptive homeostasis responses including increased Proteasome and Lon and increased stress resistance. These adaptive responses were all dependent on Nrf2 orthologues: SKN-1 in worms and Cnc-C in flies. In contrast, as with our mammalian cell culture studies above, higher levels of oxidants or redox cycling agents caused measurable damage and were less effective in engendering adaptive homeostasis.

Taken together, our results with mammalian cells in culture, worms, and flies [37, 38] are all consistent with the proposal that adaptive homeostasis operates via signal transduction, not because of damage *per se*. Adaptation can still occur in the presence of damage, but it may be less efficient or effective. Our results suggest the importance of keeping real-life exposures to potential toxicants at low levels, in order to obtain maximum adaptive homeostasis benefits. We are now perusing more detailed studies to try to define the mechanistic relationships between the concentration of a signaling agent, the degree and type(s) of toxicity that may be caused, and the ability of pathways like Nrf2 to effectuate adaptation.

Mounting evidence indicates that the adaptive stress response is not uniform between the sexes. Indeed, early studies assessing the adaptive response in mammalian cell culture almost all involve female-derived cell lines [20]. In these studies pretreatment with hydrogen peroxide showed rapid and robust activation of the stress-responsive transcriptional activator, Nrf2 [58–61], resulting in upregulation of stress inducible enzymes, including the 20S proteasome [35, 36, 62]. In addition, our previous studies of the adaptive response in the model organism, *D. melanogaster*, uncovered a female-specific adaptive response to hydrogen peroxide [39–41, 43], resulting in the upregulation of the 20S proteasome [41, 43], and a male-specific adaptation to paraquat. The present findings further demonstrate the induction of the 20S proteasome is sex and oxidant-dependent, as blockage of the 20S proteasome prevents the adaptive increase.

Unique to this study is the testing of the effects of additional redox cycling agents, specifically menadione (Vitamin K-3, 2-methyl-1,4-naphthoquinone) and DMNQ (2,3-Dimethoxy-1,4-naphthoquinone) on adaptive differences between the sexes. These quinones can continuously generate superoxide (and via dismutation, hydrogen peroxide) by accepting electrons from intracellular sources such as mitochondrial complexes I and III [37, 38] and cytochrome P450 reductase. In addition to redox cycling, menadione, which is a potent electrophile, also reacts with reduced glutathione (GSH) or the cysteine residues of proteins, to form arylation products, causing cellular damage [54]. In contrast, DMNQ continuously cycles between its reduced one-electron semiquinone state back to its oxidized state, generating the superoxide radical, which is subsequently dismutated to hydrogen peroxide by cellular superoxide dismutase enzymes [63]. A key difference between menadione and DMNQ, is the absence of arylation sites on DMNQ, resulting in an underlying difference in cytotoxicity, as first identified in male-derived hepatocytes [64]. Interestingly, the findings from the present study show that in multiple fly strains pretreatment with non-damaging amounts of DMNQ and menadione both trigger a male-specific adaptive response, as measured by increased resistance to the quinones, increased cellular content of proteasomal protein, and increased proteasomal proteolytic capacity.

The sex-specific differences in adaptive response are potentially linked to the mode of action, and/or the delivery methods, of the differing oxidants: redox cyclers versus hydrogen peroxide. Unlike introduction of bolus amounts of hydrogen peroxide via the digestive tract (through *ad libitum* feeding), redox cyclers work via the continuous generation of the superoxide radical and its dismutation into hydrogen peroxide. Though additional work is necessary to fully understand the mode of action, different pathways have been proposed for these two classes of oxidants. Specifically, non-damaging amounts of hydrogen peroxide are viewed as crucial mediators of cellular signaling. Indeed, at low concentrations, H₂O₂ has been implicated to act as an insulin mimetic [65], induce cell proliferation [66, 67], and activate various transcription factors involved in the adaptive stress response [68]. Indeed, ablation of the *Drosophila* insulin-like peptide producing neuronal cells caused increased oxidative stress resistance in females [69]. Moreover, a localized increase in H₂O₂ concentration created by membrane-associated NADPH oxidase and extracellular SOD enhances insulin-stimulated signaling through the AKT pathway [70–72], and H₂O₂ is reported to increase transcriptional binding by Nrf2 by disrupting the interaction between Nrf2 and its inhibitory factor Keap1 [73].

H_2O_2 signaling is not limited to transcription factors. This is most notable in the dynamic fluctuation in the cytosolic proteasomal pool. Under homeostatic conditions the proteasome exists in multiple forms in the cell cytoplasm, nucleus, and endoplasmic reticulum. These include the 26S proteasome, the 20S proteasome ($\pm$ 11S or PA28 regulators), and hybrid proteasomes. The 26S proteasome form, which consists of the 20S catalytic core and two 19S regulatory caps, is responsible for degrading ubiquitin-tagged proteins in an ATP-dependent manner. Adaptive concentrations of H_2O_2 trigger the sequestering of the 19S regulatory caps by HSP70, as demonstrated in the female K562 lymphoma cells and the immortalized mouse hippocampal HT22 neuronal cells (sex unknown) [74, 75]. Additionally, ECM 29, a regulatory protein involved in the assembly of the proteasome core and regulatory subunits [76], was identified, using *S. cerevisiae*, as a novel partner in 19S separation from the 20S catalytic core, under periods of oxidative stress [77]. Together, these interactions facilitate an immediate pool of available 20S proteasome for turnover of oxidized proteins during stress conditions. To further supplement the available pool of 20S proteasome, H_2O_2 stimulation also activates de novo transcriptional upregulation of the 20S proteasome subunits, providing additional protection against future oxidative insults [59]. Here, we observed that low, signaling amounts of H_2O_2 , stimulated the synthesis of increased cellular content and activity of the 20S proteasome subunits [1], and also improved survival in female *D. melanogaster*, consistent with a conserved signaling role for H_2O_2 .

In contrast, paraquat, a very strong redox cycling agent, gains its toxicity from its redox cycling capacity to continually generate superoxide through its reduction by mitochondria and cytochrome P450 reductase [78–80]. In turn, the redox cycling ability of paraquat to continually generate superoxide, has been shown to increase the expression of key antioxidant enzymes, including superoxide dismutase and catalase, both necessary in the breakdown of superoxide [81, 82]. In vitro cell culture studies showed that paraquat toxicity triggered Nrf2 activation in male-derived cell lines (not tested in female cell lines), including the rat adrenal medulla (PC12) cells [83], human bronchial epithelial cells [84], and mouse Type II alveolar epithelial cells [85]. Due to the redox cycling capacity of paraquat, it can quickly deplete cellular stores of NADPH [80]. Moreover, due to the necessity of NADPH for paraquat cycling, it has been shown to induce metabolic remodeling. Specifically, as one of the primary sources of intracellular NADPH is the pentose phosphate pathway, it is not surprising that this pathway is found to be elevated upon paraquat exposure [86]. Additionally, *D. melanogaster* studies assessing the neurological targets of paraquat, especially as a model for spontaneous Parkinson's disease, identified DJ-1, an oxidant sensitive chaperone protein, which when removed, increased male paraquat sensitivity [87].

However, paraquat's role as a signaling molecule is less well-understood. Earlier work found the activation of a group of mitogen-activated protein kinases (MAPKs), specifically the c-Jun N-terminal kinase (JNKs) [88], were activated in cell culture studies in response to extracellular stress, including heat shock (as evident in the ovarian-derived hamster cell line, CHO-K1), UV radiation (as shown in the male-derived rat adrenal medulla PC-12 cell line), and during oxidative stress, including paraquat (as demonstrated in rat dopaminergic neural N27 cells) [88–90]. The JNK pathway has been found to be activated as a means of cellular protection [91], yet under prolonged stress, may promote apoptosis [92]. Moreover, studies conducted in *D. melanogaster* larvae, found a series of genes that were regulated by the JNK

pathway [93]. Follow-up studies found that paraquat exposure resulted in the activation of the JNK signaling pathway, tested by measuring changes in four JNK-dependent genes (hsp68, gstD1, fer1HCH, and mtnA) [94]. In addition, overexpression of JNK signaling, limited to neurons, was capable of improving male survival in *D. melanogaster* [94]. The present study and the one completed in parallel [1], show that not only paraquat, but additional redox cycling agents, including DMNQ and menadione, are capable of inducing cellular signaling responses, measured through increased proteasomal proteolytic capacity.

Overall, the sex-dependent differences in response to the type of oxidant observed in *Drosophila* may be related to underlying differences in the adaptive stress response in mammalian models. Indeed, studies using rat striatal astrocytes found male-derived cells to show higher levels of oxidative stress and toxicity following hydrogen peroxide and DMNQ exposure, compared to levels in female-derived cells [95]. Furthermore, many diseases, including coronary heart disease (male-favored) [96], certain cancers (breast, thyroid, anal, female-favored; tongue, pharynx, esophagus, stomach, and liver, male-favored) [97], and Parkinson's (male-favored) [98], demonstrate an underlying elevation in reactive oxygen species in a sex-specific manner. Additionally, sex disparities in the response to oxidative stress even appear to arise at an early age, with young healthy men showing higher basal levels of oxidative stress markers compared to healthy age-matched premenopausal women [99]. Moreover, the higher rate of Parkinson's disease prevalence in men may be related to the ability of male-derived neuronal cells to have higher paraquat uptake, as it has been previously shown to be incorporated via the dopamine transporter [100]. Conversely, females may be better able to cope against oxidative stress, as findings suggest higher basal expression of antioxidant and stress-responsive cellular defenses [101, 102].

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HIGHLIGHTS

1. Sex-specific differences in the adaptive homeostatic response are oxidant-dependent
2. Redox cycling agents induce a male-specific adaptive response
3. Hydrogen peroxide induces a female-specific adaptive response
4. Sex-specific differences are consistent across wild-type *D. melanogaster* strains

Figure 1. Sex-specific sensitivity to hydrogen peroxide (H₂O₂)

(A-F) Male and female progeny were collected and placed onto 5% sucrose for 24 hours prior to hydrogen peroxide challenge [0M-8M]. (A,B) Ca-S male and female survival curves. (C,D) Or-R male and female survival curves. (E,F) w[1118] male and female survival curves.

Figure 2. Pretreatment with hydrogen peroxide results in female-specific adaptation
(A-F) Male and female progeny were collected and placed onto 5% sucrose for 24 hours prior to H₂O₂ pretreatment [0 μM-100 μM] for 8 hours. After a 16-hour recovery, flies were placed upon H₂O₂ challenge dose [4.4M]. (A-C) Males, irrespective pretreatment concentration, show no difference in survival compared to the controls. (D-F) Pretreated females showed longer survival times compared to controls upon administration of the challenge dose. Statistical difference in survival was calculated using the Log-Rank test. Statistical summary is located in Table 1 [1]. (G-K) The higher amounts of the 20S

proteasome α subunits was measured between males and females of the three strains. Females and males were pretreated with adaptive doses of H_2O_2 [0 μM -100 μM] for 8 hours, before allowed a 16-hour recovery before collection. (G,I,K) Pretreated females show increased proteasome expression. (H,J,L) Males, regardless pretreatment, show no adaptive change in the amount of proteasome. Samples were done in triplicate, protein loading was normalized to Actin-HRP, and quantified using ImageJ. Error bars denote the standard error of the mean (S.E.M) values. * $P < 0.05$ and ** $P < 0.01$, relative to the control using one-way ANOVA.

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Figure 3. Hydrogen peroxide adaptation involves female-specific increases in proteasomal proteolytic capacity

(A-F) Male and female progeny were collected and placed onto 5% sucrose for 24 hours prior to H₂O₂ pretreatment [0μM-1000μM] for 8 hours, at which point, flies were transferred to 5% sucrose for a 16-hour recovery before collection. Proteolytic capacity was assessed in whole fly lysate by degradation of the fluorogenic peptide, Suc-LLVY-AMC. (A,C,E) In all three strains, males show no change in proteolytic capacity, irrespective pretreatment. (B,D,F) Females from all three strains show higher levels of proteolysis upon H₂O₂

pretreatment. Error bars denote the standard error of the mean (S.E.M) values. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, relative to control using one-way ANOVA.

Figure 4. Sex-specific sensitivity to paraquat (PQ)

(A-F) Male and female progeny were collected and placed onto 5% sucrose for 24 hours prior to paraquat challenge [0mM-40mM]. (A,B) Ca-S male and female survival curves. (C,D) Or-R male and female survival curves. (E,F) w[1118] male and female survival curves.

Figure 5. Pretreatment with paraquat results in male specific adaptation

(A-F) Male and female progeny were collected and placed onto 5% sucrose for 24 hours prior to paraquat pretreatment [0 μ M-10 μ M] for 8 hours. After a 16-hour recovery, flies were placed upon paraquat challenge dose [30mM]. (A-C) Upon pretreatment, males survive longer compared to males receiving only the challenge dose. (D-F) Females, irrespective pretreatment, show no difference in survival upon introduction of the challenge dose. Statistical difference in survival was calculated using the Log-Rank test. Statistical summary is located in Table 2 [1]. (G-K) The adaptive change of 20S proteasome α subunit

expression was measured between males and females of the three wild-type strains. Females and males were pretreated with adaptive doses of paraquat [0 μ M-10 μ M] for 8 hours, before allowed a 16-hour recovery before collection. (G,I,K) Females show no change in proteasome expression following paraquat pretreatment. (H,J,L) Paraquat pretreated males show increased proteasome expression compared to controls. Samples were done in triplicate, protein loading was normalized to Actin-HRP, and quantified using ImageJ. Error bars denote the standard error of the mean (S.E.M) values. * $P < 0.05$ and ** $P < 0.01$, relative to the control using one-way ANOVA.

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Figure 6. Paraquat adaptation involves male-specific increases in proteasomal proteolytic capacity

(A-F) Male and female progeny were collected and placed onto 5% sucrose for 24 hours prior to paraquat pretreatment [0μM-100μM] for 8 hours, at which point, flies were transferred to 5% sucrose for a 16-hour recovery before collection. Proteasomal proteolytic capacity was assessed in whole fly lysate by degradation of the fluorogenic peptide, Suc-LLVY-AMC. (A,C,E) Males from all three strains show higher levels of proteolysis upon paraquat pretreatment. (B,D,F) All three strains, females show no change in proteolytic capacity, irrespective pretreatment. Error bars denote the standard error of the mean (S.E.M)

values. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, relative to control using one-way ANOVA.

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Figure 7. Pretreatment with DMNQ results in male specific increases in proteasomal proteolytic capacity

(A-F) Male and female progeny of the Ca-S, Or-R, and w[1118] strains were collected and placed onto 5% sucrose for 24 hours prior to DMNQ pretreatment [0μM-10μM] for 8 hours, at which point, flies were transferred to 5% sucrose for a 16-hour recovery before collection. Proteasomal proteolytic capacity was assessed in whole fly lysate by degradation of the fluorogenic peptide, Suc-LLVY-AMC. (A,C,E) Males from all three strains showed higher levels of proteolysis upon DMNQ pretreatment. (B,D,F) All three strains, females show no change in proteolytic capacity, irrespective pretreatment. Error bars denote the standard error

of the mean (S.E.M) values. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, relative to control using one-way ANOVA.

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Figure 8. Pretreatment with menadione results in male specific increases in proteasomal proteolytic capacity

(A-F) Male and female progeny of the Ca-S, Or-R, and w[1118] strains were collected and placed onto 5% sucrose for 24 hours prior to menadione pretreatment [0 μ M-10 μ M] for 8 hours, at which point, flies were transferred to 5% sucrose for a 16-hour recovery before collection. Proteasomal proteolytic capacity was assessed in whole fly lysate by degradation of the fluorogenic peptide, Suc-LLVY-AMC. (A,C,E) Males from all three strains show increased proteolysis upon menadione pretreatment. (B,D,F) All three strains, females show no change in proteolytic capacity, irrespective pretreatment. Error bars denote the standard

error of the mean (S.E.M) values. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, relative to control using one-way ANOVA.

Figure 9. Adaptive increases in proteolytic capacity are dependent upon the proteasome
 Flies of the Ca-S strain were pretreated with either no H₂O₂ or 10 μM H₂O₂. Afterwards, whole fly lysate underwent immunoprecipitation (IP) against the 20S proteasome α subunit *D. melanogaster* specific monoclonal antibody. Proteolytic capacity was compared between lysate treated with or without H₂O₂ pretreatment and lysate from IP flow-through (FT). (A) Lysate from flow-through show no change in proteolytic capacity in pretreated females. (B) Male lysate from input show no change in proteolysis, irrespective pretreatment, with flow-through showing decreased basal 20S proteolysis. Error bars denote the standard error of the

mean (S.E.M) values. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, relative to input control using one-way ANOVA.

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