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Exogenous oxidative stressors elicit differing age and sex effects in *Tigriopus californicus*

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ABSTRACT

As organisms age, cellular function declines in a time-dependent manner. Oxidative stress induced by reactive oxygen species damages cellular machinery and contributes to senescence which narrows the homeostatic window needed to maintain function and survive stress. Sex differences in longevity are apparent in many species and may be related to sex-specific homeostatic responses. Here we use the emerging aging model system *Tigriopus californicus*, the splashpool copepod, to estimate sex- and age-specific tolerances to two chemical oxidants, hydrogen peroxide and paraquat. Sex-specific tolerance was estimated for both oxidants simultaneously for 15 age-classes. As animals aged, hydrogen peroxide tolerance decreased but paraquat tolerance increased. Also, we observed no sex difference for hydrogen peroxide tolerance, while females were more tolerant of paraquat. Our results demonstrate that oxidative stressors can have dramatically different sex and age effects in *Tigriopus californicus*. These findings underscore the challenges ahead in understanding relationships among oxidative stressors, sex, and aging.

1. Introduction

Aging manifests as the time-dependent decline in cellular function resulting in compromised physiologies which elevate the risk of death (López-Otín et al., 2013). The resultant age associated progressive increase in cellular dysfunction, especially in the enzymatic synthesis of energy, contributes to an increase in reactive oxygen species (ROS) (Harman, 1956, 1992). Organisms experience oxidative stress when ROS levels exceed the reductive antioxidant capacity of intracellular chemicals and enzymes (Beckman and Ames, 1998), and oxidative stress can further damage cellular macromolecules leading to impaired function. The cellular effects of ROS tend to be dependent on quantity; for example, small increases in ROS production can sometimes promote longevity whereas high ROS levels are detrimental (Ristow and Zarse, 2010). ROS are important signaling molecules, therefore complete depletion disrupts cellular function while too high of levels result in cellular damage. *Drosophila melanogaster* selected for increased oxidative stress resistance showed increased life span (Arking et al., 2000; Rose et al., 1992), and vice versa (Harshman and Haberer, 2000) indicating a positive relationship between longevity and oxidative stress resistance. Further, in *Caenorhabditis elegans*, single-gene mutations that increase life span typically also increase oxidative stress resistance (Honda and Honda, 1999; Murakami and Johnson, 1998). Typically, oxidative stress tolerance correlates with life span and life span extending single gene mutations increases oxidative stress tolerance. However, studies have also demonstrated the opposite (e.g. Csiszar et al., 2007), indicating a complex relationship between ROS and life span which has been difficult to generalize across species (Shields et al., 2021).

Adaptive homeostasis is a mechanism wherein organisms maintain function by rapidly altering their homeostatic window to withstand damaging molecules or stressful conditions (Davies, 2016; Pomatto and Davies, 2018). As organisms age, the ability to modify the adaptive homeostatic response to stressors weakens and therefore the ability to mitigate damage declines (Pomatto et al., 2019). Sex also influences the homeostatic response (Pomatto et al., 2018; Pomatto et al., 2017a, 2017b). In *D. melanogaster*, females pretreated with low levels of hydrogen peroxide (H_2O_2) survived a subsequent toxic H_2O_2 challenge better than untreated females, while this was not observed in males (Pomatto et al., 2017b). In turn, males pretreated with low levels of the redox cycler paraquat had increased survival during a subsequent toxic paraquat challenge, while this was not observed in females (Pomatto et al., 2017b). These results underscore the importance of sex as a biological determinate of oxidative stress resistance (Tower et al., 2020). To our knowledge, the contrasting sex effects of hydrogen peroxide and

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paraquat have not been demonstrated outside of *D. melanogaster*.

Here, we use the aquatic invertebrate *Tigriopus californicus* to estimate the sex and age specific effects of these same two exogenous oxidative stressors. *T. californicus* occupies the supralittoral splash pools from northern Baja California to Southern Alaska (Edmands, 2001) which can experience high daily environmental fluctuations (Leong et al., 2018). Adult males perform a mate-guarding behavior where males use modified antennae to clasp virgin females until they become receptive. The male will then mate with the female and release her (Burton, 1985). Females mate once and iteratively produce clutches sired by a single male (Burton, 1985). Mitochondria in this species are maternally inherited (Lee, 1993). *T. californicus* lacks canonical sex chromosomes and instead sex is polygenic where multiple independent loci throughout the genome determine sex as a threshold quantitative trait (Alexander et al., 2015; Ar-Rushdi, 1958; Voordouw and Anholt, 2002). Male-female divergence occurs across the genome and is not restricted to sex chromosomes, but members of the same sex within a population can show variation at these sex determining regions. Fixed male-female differences are therefore expected to be minimal under polygenic sex determination. In this alternative sex determination system, sex-specific phenotypes are not complicated by the presence of sex chromosomes which influence sex differences in life span (Xirocostas et al., 2020) and can result in dosage compensation (Disteche, 2012).

Even without sex chromosomes, sex differences are pervasive in *T. californicus*. Females are larger and show higher acute stress tolerance to multiple stressors including heat (Foley et al., 2019; Kelly et al., 2012; Willett, 2010), salinity, copper, and bisphenol A (Foley et al., 2019), while males have a longer life span, increased DNA damage with age and a lower mitochondrial DNA content (Flanagan et al., 2021). Under long term (Li et al., 2019) and short term (Li et al., 2020) oxidant exposure, *T. californicus* males differentially expressed more genes than females, and the majority of gene expression variance was partitioned between the sexes. These differently expressed genes include those known to respond to oxidative stress including superoxide dismutase and glutathione S-transferase, which both showed greater upregulation in males (Li et al., 2019, 2020).

In the current study, we use *T. californicus* to investigate age and sex differences in acute tolerance to two exogenous oxidative stressors, H_2O_2 and paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride). Intracellularly, paraquat reacts with NADPH oxidase enzymes and oxygen in the mitochondria and at the cell membrane to produce superoxide ions, which are in turn enzymatically dismutated into H_2O_2 (Cochemé and Murphy, 2008). Paraquat is an effective redox cycler causing continuous production of superoxide ions, while H_2O_2 induced oxidative stress occurs only through chemical exposure and does not react with cellular chemicals to produce additional oxidants.

2. Methods

2.1. Sample collection and experimental design

Wild copepods were collected from San Diego, CA (25 June 2018) and taken to the lab at the University of Southern California. The wild collections were maintained in 1 L beakers containing triple filtered (37 μm) natural seawater collected from Wrigley Marine Science Center, Catalina Island, CA. Animals were fed the blue-green alga Spirulina (Nutraceutical Science Institute, USA) and Tetramin fish food (Tetra Holding Inc., USA) mixture (0.01 % MV) and incubated at 20 °C on a 12:12 light:dark cycle throughout the experiment.

The experiment was designed so that copepods of different ages were assayed at the same time, with cohorts split between experimental treatments (Fig. 1). To generate cohorts of copepods with known age, 90 gravid females with orange egg sacs were isolated and held at 20 °C overnight to allow eggs to hatch. Females with orange eggs were targeted because the orange coloration is indicative of late-stage development just prior to hatching. Larvae were incubated at 20 °C on a 12:12 light: dark cycle and were allowed to develop for four weeks or until gravid females were observed. After we observed gravid females, to ensure age identity, all animals were transferred weekly to remove newly hatched larvae. Transfers were repeated weekly to generate 15 age-classes ranging from four to 22 weeks post-hatching with a four-week gap between the 17th and 22nd week cohort. All ages overlapped and oxidant exposure for all age classes was performed at the same time. All experimental animals were cultured in the same incubator at 20 °C on a 12:12 light: dark cycle.

This range of ages was chosen based on previous longevity estimates where 10 % of animals survive until 22 weeks of age after reaching sexual maturity (Flanagan et al., 2021). Four weeks was chosen as the lower limit for the age range because juvenile *T. californicus* stages (copepodite stages I–V) occurring earlier in life have not developed

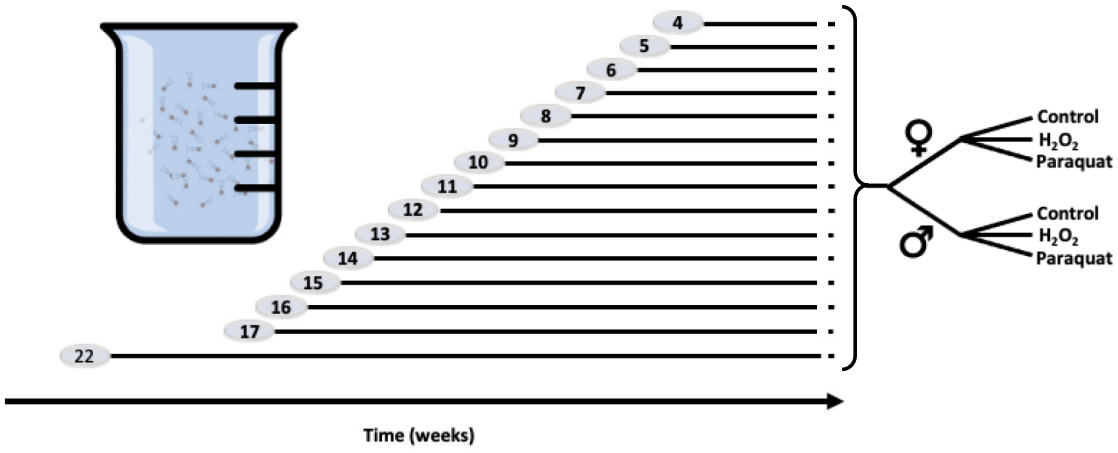

Fig. 1. Experimental design to estimate sex- and age-specific oxidative stress tolerance for two oxidants, hydrogen peroxide and paraquat. Weekly, 90 gravid females with developmentally late-stage egg sacs were isolated from a stock population maintained in a beaker. After isolation, females were given 24 h for egg sacs to hatch. The females were then removed and placed back into the stock population beaker. This was repeated weekly to generate 15 age-classes spanning from four to 22 weeks post-hatching. Simultaneously for all age-classes, the sexes were separated and exposed to experimental conditions, including benign control conditions, to estimate hydrogen peroxide and paraquat tolerance. Dashes indicate treatment exposures were performed for all age-classes.

diagnostic sexual structures making it difficult to distinguish males from females (Egloff, 1966). Additionally, no sex-biased mortality occurred in the San Diego population during the first 28 days following hatching (Flanagan et al., 2021).

To estimate oxidative stress tolerance age- and sex-specifically, each age-matched cohort was first split based on sex. *T. californicus* males can be identified by a diagnostic modified antenna under a dissecting scope at 40×. For each sex of each age, tolerance was estimated by exposing three replicates of seven to ten individuals to either benign control conditions containing triple filtered seawater, 1.2 mM H_2O_2 or 0.31 mM paraquat, two chemical oxidants. These oxidant concentrations were based on mean 24 h LC50 (the oxidant concentration required to kill 50 % of the population) for wild animals from the San Diego population (unpublished data). Further, previous work in *T. californicus* has indicated these concentrations of both oxidants alter the gene expression of proteases and antioxidant genes including glutathione S-transferase (Li et al., 2020). The oxidant concentrations employed are above those in natural conditions but were chosen to induce an oxidative stress response under acute conditions.

Oxidant exposure occurred in 90 mm diameter Petri dishes with 20 mL of seawater. To document the mortality, animals were observed every 4 h up to 32 h of exposure at which point mortality was observed intermittently up to 120 h of exposure (timepoints for mortality observation; 0, 4, 8, 12, 16, 20, 24, 28, 32, 44, 48, 52, 72, 76, 80, 96, 100, 104, 120). This method of oxidant exposure is different from those typically employed in terrestrial systems where oxidants are administered through ingestion (see Arking et al., 1991) because here the oxidant is a component in the respiratory medium (but see Landis et al., 2004).

2.2. Statistical analysis

To estimate sex- and age-specific oxidant tolerance, the proportion of surviving individuals observed for each hour of exposure and replicate was fit to a generalized linear mixed effects model with a binomial error distribution in R (R Core Team, 2021) using the *lme4* package (Bates et al., 2015), and models were fit by maximum likelihood. Treatment showed a significant effect on the proportion of surviving individuals (Table S1) and because the load of oxidative stress is not equivalent among our two pro-oxidant treatments, they were fit independently. The proportion of alive individuals in each replicate was fit to a generalized linear mixed effects model with fixed effects of sex, age, and hour and their two-way interactions with a random effect of replicate (subject) within exposure hour (*glmer*, Proportion alive ~ Sex + Age + Hour + Sex:Hour + Age:Hour + Sex:Age + (Hour|Subject). The interaction terms were sequentially dropped to determine the minimum adequate model by selecting the model with the lowest Akaike's information criterion (AIC) and Bayesian information criterion (BIC) for each oxidant exposure (Table S2).

Survival data was fit to a non-parametric Kaplan-Meier survival model to investigate the effects of age and sex on survival within each treatment. Data are right censored and animals still alive at the end of the treatment exposure were included in the survivorship analysis. To estimate the age effect, we divided ages into quintiles (Q1 4–6 weeks; Q2 7–9 weeks; Q3 10–12 weeks; Q4 13–15 weeks; Q5 16, 17, and 22 weeks) in R (R Core Team, 2021) and fit survival models using the *survfit()* function in the survival package (Therneau, 2022). Pairwise survival differences were estimated using the *pairwise survdiff()* function in the *survminer* R package (Kassambara et al., 2021) with correction for multiple tests using the method of Benjamini and Hochberg (1995).

3. Results

In all, survival was estimated for 2571 animals which included three replicates of seven to ten individuals for each age, sex, and treatment including benign control conditions. Mortality was not observed for all individuals and there were 334 and 156 animals surviving at the end of the 120 h exposure to H_2O_2 and paraquat respectively.

The control treatment experienced mortality and 17 % died after 120 h (n = 857). While this mortality was higher than expected, the proportion alive for the two pro-oxidant treatment groups was lower than the control indicating both oxidant treatments effectively increased mortality (Fig. 2; Fig. 2; Table S1). The best fit generalized linear mixed effects models were different for the two pro-oxidant treatments. The best fit model for H_2O_2 included the fixed effects of age, sex, and hour as well as the interactive effects of age and hour and sex and hour (Proportion alive ~ Sex + Age + Hour + Age:Hour + Sex:Hour; Table S2A), while the best fit model for paraquat included the same fixed effect and only the interactive effect of sex and hour (Proportion alive ~ Sex + Age + Hour + Sex:Hour + Hour|Subject; Table S2B). Here we are particularly interested in the age and sex effects within each treatment and not the relationship between the two pro-oxidant treatments, therefore we estimated the age and sex effects independently for each oxidant (Table 1). When animals spanning 18 weeks of age were exposed to H_2O_2, there was no difference between the two sexes (Table 1A; *glmm*, z-value = −0.63, p-value = 0.529) while age significantly affected the proportion alive (Table 1A; *glmm*, z-value = 36.86, p-value <0.001) and interacted with exposure hour wherein the proportion alive decreased with increasing age (Table 1A; *glmm*, z-value = − 0.002, p-value <0.001) (Fig. 2; Fig. S2). The interaction of age and hour is driven by the H_2O_2 sensitivity of young males at four weeks of age (Fig. S1). For the paraquat treated animals, sex affected the proportion alive (Table 1B; *glmm*, z-value = 3.626, p-value <0.001) and was dependent on the exposure hour (Table 1B; *glmm*, z-value = −0.138, p-value <0.001) where females were more tolerant than males (Fig. S3). Additionally, the proportion alive over the course of paraquat exposure increased for older animals (Table 1B; *glmm*, z-value = 0.0125, p-value <0.001).

In an alternative modeling approach, survival was fit to the Kaplan-Meier model to estimate the effects of sex and age. Ages were divided into quintiles, and similarly to the results of the generalized linear mixed effects model, sex did not affect H_2O_2 survival (p-value = 0.58), and H_2O_2 survival decreased with increasing age quintiles (Fig. 3). Sex did affect paraquat survival where females were more tolerant (p-value <0.001), and paraquat tolerance changed with age (Fig. 3). For females, paraquat tolerance increases with age while for males the tolerance peaked in the second quintile but was lowest for the oldest quintile (Fig. 3).

4. Discussion

Acute exposure to two oxidants resulted in differing age and sex effects where males were more sensitive to paraquat while we detected no sex difference for H_2O_2 tolerance. For females, paraquat tolerance increased with age whereas H_2O_2 tolerance decreased with age, and in males, H_2O_2 tolerance decreased with age while paraquat tolerance initially increased to peak in the seven- to nine-week-old quintile.

The decrease in paraquat sensitivity for older animals was unforeseen based on the expected age-related decline in physiological function and homeostasis (López-Otín et al., 2013). This expected age-related decline has been specifically demonstrated for paraquat tolerance in *Drosophila* (Belyi et al., 2020). In our study, H_2O_2 tolerance exhibited the expected age-associated decline in tolerance while the pattern was reversed for paraquat, even for males and females originating from the same egg sacs and assayed simultaneously. This indicates that these two commonly used oxidative stressors elicit markedly different responses, at least in *T. californicus*. Hydrogen peroxide exposure results in a static load of oxidative stress while paraquat, due to its activity as a redox cycler, continuously generates superoxide radicals. Both H_2O_2 and paraquat induce the expression of key antioxidants (Abrashev et al., 2011) whereas only paraquat exposure depletes NAD(P)H (Forman et al., 1980; Suntres, 2002). While further experimentation is required, we hypothesize the opposite age-associated tolerances in both sexes for the two oxidative stressors may be related to membrane depolarization

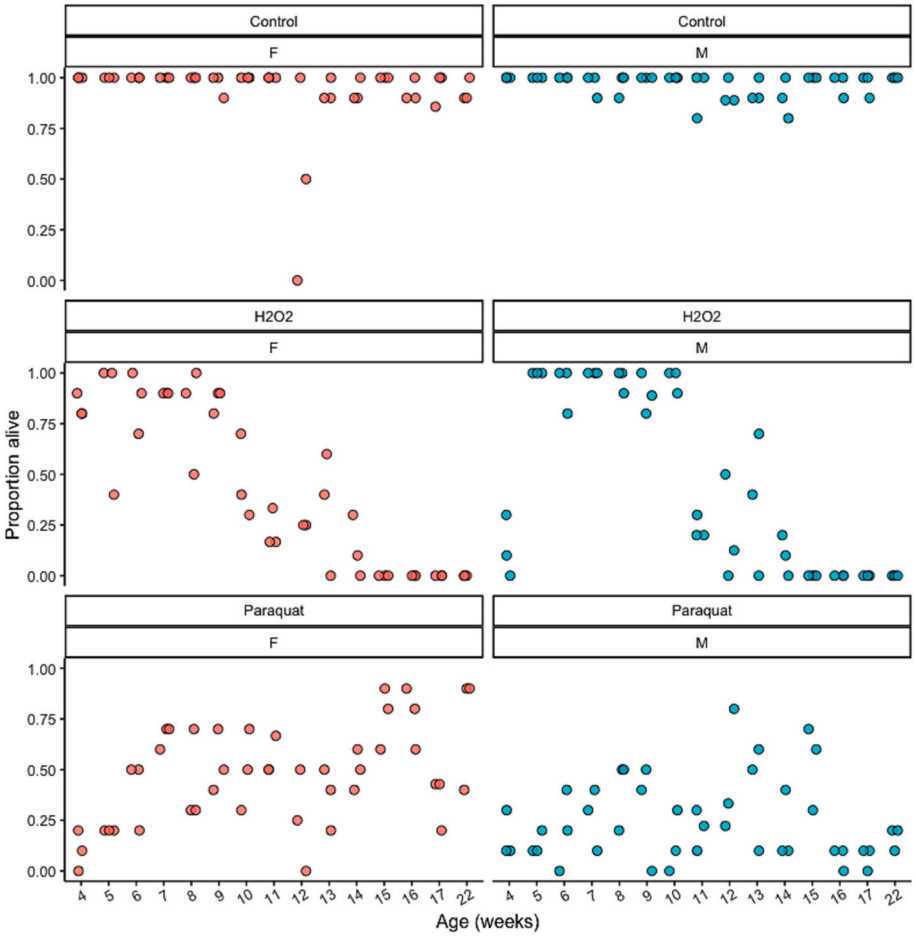

Fig. 2. The sex-specific (blue – male (M), red – female (F)) oxidative stress tolerance for all age-classes after 48 h of exposure to hydrogen peroxide (H_2O_2). Points represent the proportion of live individuals following 48 h exposure for each of three replicates per age and treatment condition. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Results of the generalized linear mixed effects model estimating the effect of sex and age on the proportion alive independently for both hydrogen peroxide (A) and paraquat (B) with random effects of effects of replicate and exposure hour. Significant effects are shown in bold.

	Estimate	Std. Error	Z-value	P-value
A) H_2O_2 (proportion alive ~ Sex + Age + Hour + Age:Hour + Sex:Hour)				
Sex (male)	−0.212	0.348	−0.63	0.529
Age	**0.036**	**0.001**	**36.86**	**<0.001**
Hour	**0.102**	**0.001**	**100.71**	**<0.001**
Age:Hour	**−0.002**	**0.001**	**−26.08**	**<0.001**
Sex(male):Hour	0.001	0.001	1.19	0.234
B) Paraquat (proportion alive ~ Sex + Age + Hour + Sex:Hour)				
Sex (male)	**3.626**	**0.71**	**4.961**	**<0.001**
Age	**0.0125**	**0.04**	**3.96**	**<0.001**
Hour	**−0.08**	**0.006**	**−14.133**	**<0.001**
Sex (male):Hour	**−0.138**	**0.019**	**−7.147**	**<0.001**

because paraquat uptake requires a membrane potential (Cochemé and Murphy, 2008) and may be related to age-associated declines mitochondrial respiration rates (Ferguson et al., 2005).

Notably, the electron transport chain in *T. californicus* differs from many other organisms, including *Drosophila*, due to an alternative pathway in the mitochondrial respiratory system. In addition to the mitochondrial proteins responsible for oxidative phosphorylation (Complexes I–V), *T. californicus* (along with a variety of other taxa) has a nuclear gene which encodes for the enzyme alternative oxidase (AOX) (Tward et al., 2019). Like nuclear encoded oxidative phosphorylation enzymes, alternative oxidase localizes to the inner mitochondrial membrane but allows for electrons to bypass Complexes III and IV and yields no contribution to the proton motive force because the energy is released as heat (McDonald, 2008). AOX reduces the mitochondrial membrane potential in non-chemically inhibited human cell lines (Cannino et al., 2012). In the tardigrade *Hypsibius exemplaris* natively expressing AOX, mitochondrial membrane potential was maintained when mitochondria respiratory Complexes III and IV were chemically inhibited (Wojciechowska et al., 2021). These results in the tardigrade are concordant with findings in non-natively AOX expressing mouse models (Szibor et al., 2020). The presence of AOX in *T. californicus* represents a respiratory pathway that may influence age-related mitochondrial membrane potential, though more work is necessary to understand the relationship.

Sex differences for abiotic stressors are common in *T. californicus* where females are more tolerant to a multitude of abiotic stressors including high salinity, low salinity, and high temperature (Foley et al., 2019). Experimental animals were derived from the wild San Diego population and previous work has shown males from this population consistently outlive females (Flanagan et al., 2021). Further, males were found to have a lower mitochondrial DNA content, and oxidative DNA damage was found to increase with age in males, but not in females (Flanagan et al., 2021). Sex in *T. californicus* is determined polygenically where multiple independent loci segregate among males and females to determine sex (Alexander et al., 2015; Ar-Rushdi, 1958; Voordouw and

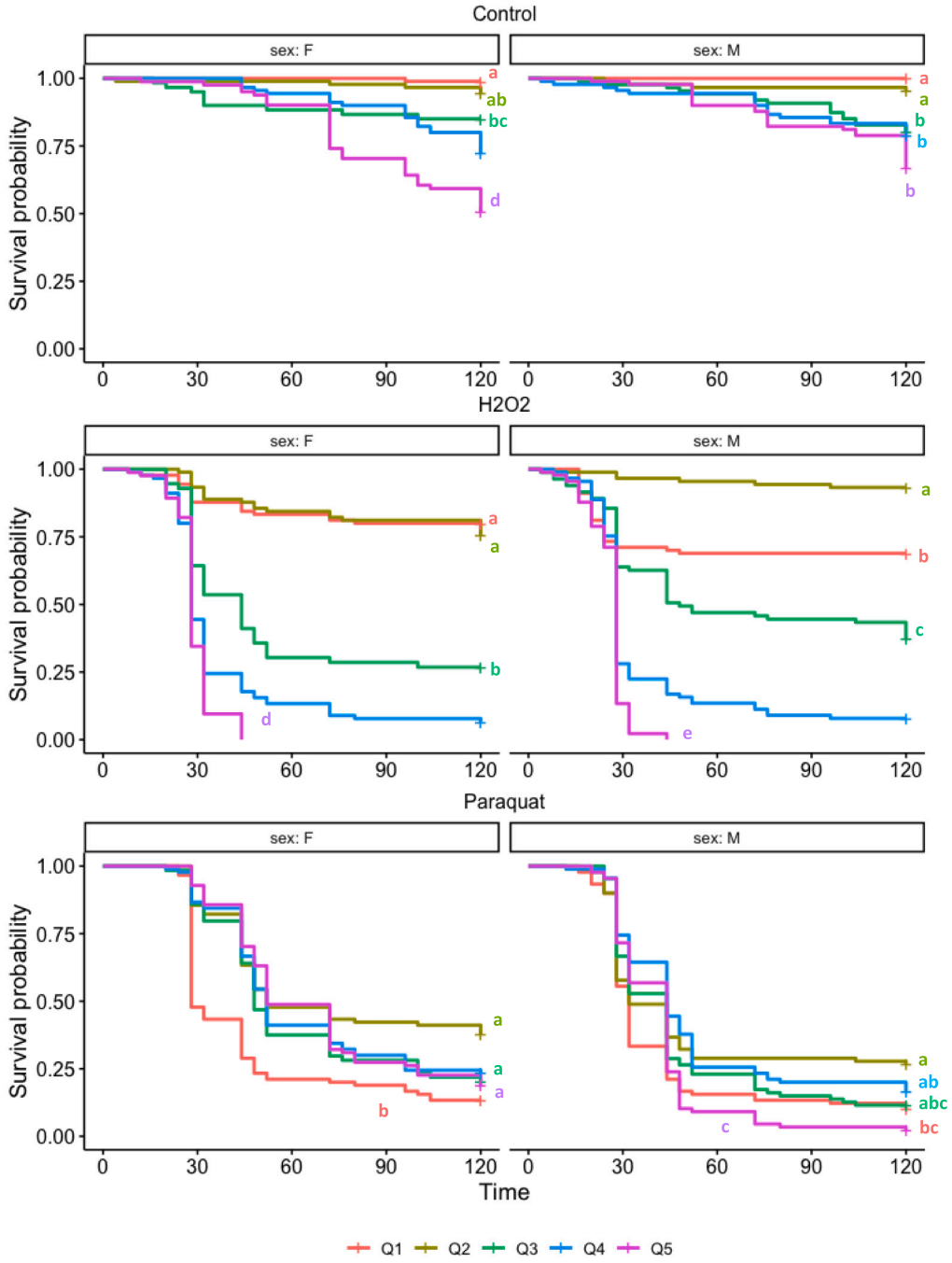

Fig. 3. Survival curves estimated using the Kaplan-Meier method for each sex within each oxidant treatment for age quintiles. Letters (a, b, c, d, e) represent pairwise differences using Log-Rank test ($p < 0.05$) with the Benjamini and Hochberg (1995) *p*-value correction methods. Age classes are divided into quintiles (Q1 4–6 weeks; Q2 7–9 weeks; Q3 10–12 weeks; Q4 13–15 weeks; Q5 16, 17, and 22 weeks). See Table S3 for sample sizes.

Anholt, 2002). Much of our understanding of sex-specific aging has focused on explanations involving sex chromosomes, particularly the unguarded x hypothesis which explains why the heterogametic sex commonly dies younger (e.g. Xirocostas et al., 2020). However, comparative studies across taxa with a range of sex-determining mechanisms (reviewed in Bronikowski et al., 2022) can allow the effects of sex chromosomes to be disentangled from other contributing factors, including sex-determining loci, mitochondria and hormonally-mediated sexual development. *T. californicus* is a particularly good model for assessing alternative drivers of sex-bias, since it exhibits substantial sexual dimorphism in the absence of heteromorphic sex chromosomes.

Our finding of sex differences in paraquat tolerance is consistent with work in *Drosophila*, where the genetic architecture for paraquat susceptibility (assayed via climbing ability) appears to be largely sex-specific, as only two out of nearly two million polymorphisms associated with this trait are shared between males and females, while over 90 % of the associated genes were sex-specific (Lovejoy et al., 2021). Additionally, variation in paraquat susceptibility in males was associated with over twice as many genes as in females (Lovejoy et al., 2021). In *T. californicus*, under short-term exposure to paraquat and H_2O_2 using the same oxidant concentrations in this study, males and females have different transcriptional profiles where males differentially express

more than four times as many genes in response to both oxidants, including up-regulation of more antioxidant genes, heat shock proteins and protease genes (Li et al., 2020). The contrasting sex-specific effects of the same two oxidants in *Drosophila and Tigriopus* underscore the importance of addressing sex as a biological variable across different species.

In sum, exogenous oxidative stressors elicited differing age and sex effects. Old male and female animals were more sensitive to H_2O_2 while both sexes derived from the same families were more resistant to paraquat when compared with younger animals, and overall females were more resistant to paraquat. Here, we used only a single concentration of both H_2O_2 and paraquat and patterns may change with differing oxidant concentrations, and, like all studies observing phenotypes of old animals, the animals alive in the older age-classes are the result of survivorship bias. Although further work is required to identify the mechanism, our findings that two chemical oxidants cause opposing age effects, as well as contrasting sex effects, illustrate some of the challenges ahead in understanding the relationships among oxidative stress, sex, and aging.

CRediT authorship contribution statement

Ben A. Flanagan: Conceptualization; data curation; formal analysis; investigation; writing – original draft; visualization; project administration. **Elaine H. Huang**: Methodology, data curation, investigation. **Suzanne Edmands**: Conceptualization; methodology, writing – review and editing; supervision; funding acquisition.

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Declaration of competing interest

We have no competing interests to declare.

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Data statement

Data and R scripts are publicly available on the Zenodo repository [dataset] (Flanagan et al., 2022).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exger.2022.111871.

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