Ageing differently: Sex-dependent ageing rates in *Daphnia magna*Julia Constantinou^{a,*}, Jack Sullivan^{b,c}, Leda Mirbahai^{d,*}^a School of Biosciences, University of Birmingham, Birmingham B15 2TT, UK^b MRC-ARUK Centre for Musculoskeletal Ageing Research, Institute of Inflammation and Ageing, University of Birmingham, Birmingham B15 2TT, UK^c NIHR Surgical Reconstruction and Microbiology Research Centre, University Hospital Birmingham, Birmingham B15 2WB, UK^d Warwick Medical School, University of Warwick, Coventry CV4 7AL, UK

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ABSTRACT

Ageing is defined as the gradual decline of normal physiological functions in a time-dependent manner. Significant progress has been made in characterising the regulatory processes involved in the mechanisms of ageing which would have been hindered without the use of model organisms. Use of alternative model organisms greatly diversifies our understanding of different factors underpinning the ageing process and the potential translation for human application. Unique characteristics make *Daphnia* an attractive model organism for research into mechanisms underlying ageing, such as transparent body, short generation time, well-characterised methylome, regenerative capabilities and available naturally occurring ecotypes. Most interestingly, genetically identical female and male *Daphnia* have evolved different average lifespans, providing a unique opportunity for understanding the underlying mechanisms of ageing and regulation of lifespan. Investigating sex differences in longevity could provide insight into principal mechanisms of ageing and lifespan regulation. In this study we provide evidence in support of establishing genetically identical female and male *Daphnia* as unique and valuable resources for research into mechanisms of ageing and begin to delineate the mechanisms involved in sex differences in lifespan. We identify significant differences between genders in physiological markers such as lifespan, growth rate, heart rate and swimming speed in addition to molecular markers such as lipid peroxidation product accumulation, thiol content decline and age-dependent decline in DNA damage repair efficiency. Overall, our data indicates that investigating sex differences in longevity in the clonal organism *Daphnia* under controlled laboratory conditions can provide insight into principal mechanisms of ageing and lifespan regulation.

1. Introduction

Ageing is the gradual decline in the normal physiological functions in a time-dependent manner, affecting all biological systems, such as molecular interactions, cellular function, tissue structure and systemic physiological homeostasis. The adverse effects of this decline partly manifests as an increased incidence of age-related diseases and illnesses such as cancer, cardiovascular diseases, Alzheimer's and type 2 diabetes (He and Jasper, 2014). With the world's older population (age 60 and over) continuing to grow at an unprecedented rate, it is expected that the number of people age 60 and above could more than triple by 2100 compare to 2017 (United Nations, 2017). Therefore, the socio-economic impact of an ageing population has led to significant emphases on understanding different aspects of the ageing process in the hope to

promote “healthy ageing” (Valenzano Dario et al., 2017).

There are > 350 theories about the mechanisms of age related changes (Vina et al., 2007) with conflicting and supportive evidence presented for each theory, including senescence theory of ageing (Weismann, 1882), Free Radical Theory of Ageing (FRTA) (Harman, 1956) and DNA damage theory of ageing (Gensler and Bernstein, 1981). Broadly the theories of ageing can be divided into two main categories of programmed ageing and ageing caused by the accumulation of damage (Sergiev et al., 2015). However, no one theory is sufficiently able to explain the process of ageing, and they often contradict one another. This demonstrates the complex nature of ageing and contribution of multiple mechanisms, as cause or as consequence, to the ageing process. For example, oxidative stress induced-damage to the cellular macromolecules has long been highlighted as a fundamental

Abbreviations: FRTA, Free Radical Theory of Ageing; ROS, reactive oxygen species; GSH, glutathione; GSSG, glutathionine disulphide; DSBs, double strand breaks; CtBP1, C-terminal binding protein 1; dHJ, double-Holliday junction; SDSA, synthesis-dependent strand annealing

* Corresponding authors.

E-mail addresses: JKC335@student.bham.ac.uk (J. Constantinou), Leda.Mirbahai@warwick.ac.uk (L. Mirbahai).

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mechanism involved in most senescence-associated alterations (Praticò, 2002) and ageing process. The progressive and irreversible accumulation of oxidative damage can contribute to impaired physiological function, increased incidence of disease and can potentially impact the ageing process (Chen et al., 2007).

During oxidative stress, an imbalance between the generation of reactive oxygen species (ROS) and their detoxification leads to various oxygen radicals to escape and damage a range of macromolecules, including mitochondrial and nuclear genomic DNA, lipids and proteins, leading to genome instability and protein/lipid peroxidation. It has been widely demonstrated that there is an age-associated increase in the steady-state concentrations of lipid peroxidation. Oxidised lipid products are linked to initiation and/or mediation of some of the aspects of the ageing process. Furthermore, it is accepted that lipid peroxidation propagates more lipid peroxidation, promoting further damage (Wanjala et al., 2017). The damaging effects of lipid peroxidation include altering cellular structural integrity, fluidity and permeability as well as loss of bio-membrane function and have been noted to generate potentially toxic products. These changes have been identified as key contributing factors to development of many age-related diseases including cancer, cardiovascular disease and Alzheimer's. However, although ageing may involve damage to various macromolecules, for those that can be replaced by their fast turnover, damage may not accumulate and therefore may not be critical. DNA, on the other hand, is the prime information molecule of the cell and nuclear DNA in particular must last the lifetime of the cell. Therefore, DNA damage represents a critical threat to cell function. If DNA damage is severe or its accumulation exceeds its elimination by DNA repair mechanisms, cellular senescence or apoptosis will occur and this may contribute to cellular dysfunction and the ageing process (Freitas and de Magalhaes, 2011; Niedernhofer et al., 2018).

The increase in macromolecules peroxidation and genome instability can be partly driven by depletion of antioxidant defence mechanisms, such as thiol-containing glutathione, as well as decline in the efficiency of the DNA damage repair machinery as a function of age, contributing to the ageing process and premature age-related syndromes (Delabaere et al., 2017). The antioxidant glutathione in its reduced form (GSH) is the most abundant non-protein thiol and acts as a substrate for glutathione peroxidase. The latter is the principal mechanism of hydrogen peroxide and lipid hydroperoxide reduction and also plays a role in catalysing the reduction of hydrogen peroxide by peroxidoxins. The reaction of GSH with hydrogen peroxide results in the production of its disulphide form; glutathionine disulphide (GSSG) (Dickinson and Forman, 2002). During ageing, the levels of the antioxidant glutathione appear to decline in a number of tissues, thereby putting cells at increased risk of oxidative stress. The observed age-related decline is possibly partly due to increased oxidative load over time (Giustarini et al., 2006). Furthermore, reduced age-dependent repair capacity can contribute to the ageing process. In both ageing and premature ageing syndromes common characteristics include higher incidence of DNA double strand breaks (DSBs). The preferential repair mechanism for DSBs is homologous recombination (HR), whereby accurate repair of the break site is fulfilled using a homologous DNA sequence as a template which is able to correctly restore the lost information. Initiation of HR repair is facilitated by the Mre11-RAD50-Nbs1 complex in addition to the C-terminal binding protein 1 (CtBP1) interacting protein (CtIP). This complex drives 5'-3' sectioning, resulting in a 3' protruding single-stranded DNA. Following this, RAD51 is recruited to the site which drives recognition of homology and strand exchange between homologous templates. RAD51 binds to the single-stranded DNA and forms a joint molecule to the repair template resulting in the formation of a D-loop. Subsequently, RAD51 is removed and further processing of the break can take two paths; formation of a double-Holliday junction (dHJ) or synthesis-dependent strand annealing (SDSA). dHJ produces two Holliday junctions by extending the D-loop to result in either crossover or non-crossover products, whereas

SDSA produces only a non-crossover product by separating the newly synthesised sequence from the template and re-ligating to the original break site. dHJ and SDSA are used predominantly for meiosis and mitosis respectively. It was found that in older flies high levels of RAD51 were expressed for longer periods of time following DSB induction. It is believed this excessive RAD51 recruitment to the break site and inhibited dissociation of RAD51 blocks the progress of HR resulting in less effective repair of damaged sites (Delabaere et al., 2017).

In the past two decades, significant progress has been made in identifying and characterising the regulatory processes involved in the mechanisms of ageing. It is important to highlight that these advances would have been hindered without the use of model organisms. Majority of the molecular mechanisms underpinning ageing processes were characterised through the use of the yeast *Saccharomyces cerevisiae*, the nematode worm *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, and the house mouse *Mus musculus* (Evans et al., 2008; Valenzano Dario et al., 2017; Kapahi et al., 2017; Ziehm et al., 2017; de Magalhaes et al., 2017). However, the use of "canonical" model organisms in the field of ageing is not without limitations. For example, the unicellular nature of yeast prevents systematic ageing studies. Both adult nematode worms and flies are mostly composed of post-mitotic cells, limiting their use as a model for stem cell ageing studies (Tissenbaum and Guarente, 2002). Furthermore, their DNA methylation machinery is either absent or not comparable to humans, thus limiting their use for investigating the role of DNA methylation in the ageing process. Whilst the maximum lifespan of mouse (> 3 years) requires considerable time and resources for lifespan studies (Valenzano Dario et al., 2017). Therefore, use of other complementary organisms, such as the African turquoise killifish (*Nothobranchius furzeri*) (Valenzano et al., 2011; Valenzano et al., 2015; Harel and Brunet, 2015; Harel et al., 2015), the planarian flatworm (*Dugesia tigrina*) (Dasheiff and Dasheiff, 2002), the naked mole rat (*Heterocephalus glaber*) (Buffenstein, 2005; Andziak et al., 2006; Buffenstein, 2008; Pérez et al., 2009; Edrey et al., 2011; Ruby et al., 2018), and the water flea (*Daphnia* spp.) (Barata et al., 2005; Iampolskiĭ and Galimov, 2005; Pietrzak et al., 2010b; Dudycha and Hassel, 2013; Kim et al., 2013; Schumpert et al., 2014; Schumpert et al., 2015; Schumpert et al., 2016; Hearn et al., 2018b; Hearn et al., 2018a), will greatly diversify our approach and our understanding of different factors underpinning the ageing process and the potential translation for human application (Buffenstein et al., 2008).

Daphnia spp. are fresh-water branchiopods and a recognised model organism by the U.S. National Institutes of Health (Ebert, 2011). It has served as a model organism in various fields of research, including ecotoxicology, ecology, and population genetics for over a century and grows in importance for molecular studies involving neurobiology (McCoole et al., 2012; Weiss et al., 2015; Toyota et al., 2015) and the biology of ageing (Pietrzak et al., 2010b; Latta et al., 2012; Dudycha and Hassel, 2013; Lohr and Haag, 2015; Schumpert et al., 2015; Asselman et al., 2016). Several unique characteristics makes *Daphnia* an attractive model organisms for research into ageing and regulation of lifespan, such as transparent body, short generation time, well-characterised methylome (Kvist et al., 2018), regenerative capabilities with continued cell renewal and proliferation throughout their lifespan, availability of naturally occurring ecotypes with diverse lifespans ranging from a few days to several months. The availability of naturally occurring closely related subtypes will enable interbreeding and producing viable offspring with diverse lifespans (Ebert, 2005; Benzie, 2005; Asselman et al., 2016; Ram and Costa II, 2018). Moreover, *Daphnia* reproduces via cyclic parthenogenesis, allowing the generation of large clonal populations and providing a unique setup for delineating genetic and epigenetic factors contributing to the ageing process as well as enabling investigation into the impact of different environmental conditions and treatments on lifespan and ageing rate. Additionally, a genetically static population allows for phenotypic characterisation of single gene knock outs for statistically relevant sample sizes. The latter

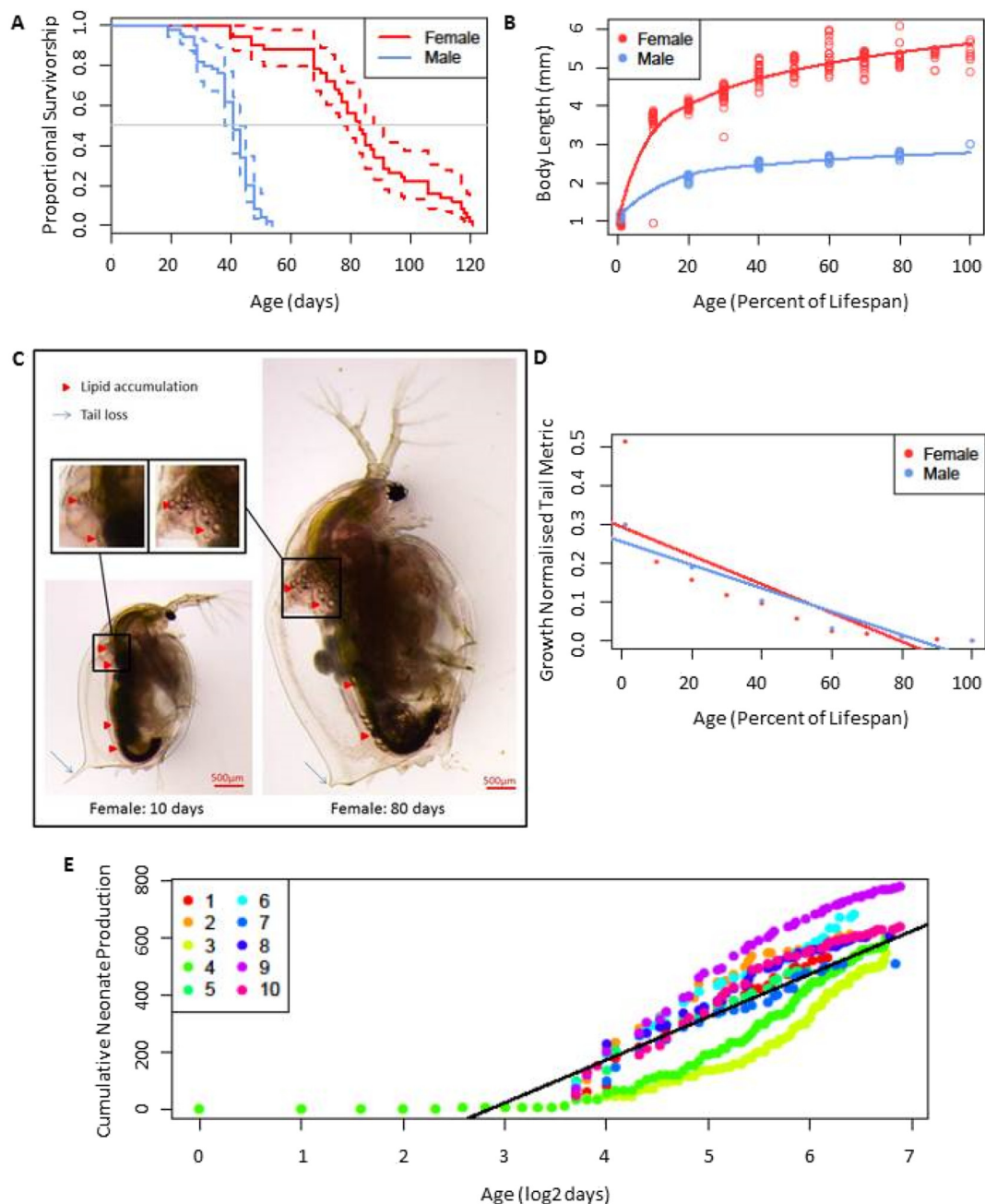


Fig. 1. Life history results for female and male *Daphnia magna* Bham 2. A) Kaplan-Meier survival curves for female (red, $n = 50$) and male (blue, $n = 25$) *D. magna* Bham 2 show significant difference in proportional survivorship (log-rank test $p = 0.0002$; 95% confidence intervals (CI) are shown for the survival curves with red and blue dash-lines). B) Growth stabilises at approximately 40% of lifespan in both females (red, $n = 25$) and males (blue, $n = 25$) *D. magna* Bham 2, however females show an overall larger body length compare to males (Log₁₀ linear regression model: significant female growth with age $R^2 = 0.94$, $p = 2.22 \times 10^{-16}$; significant male growth with age $R^2 = 0.98$, $p = 2.22 \times 10^{-16}$). C) Representative images of young (10 days) and old (80 days) female *D. magna* Bham 2 showing visible signs of ageing, including accumulation of lipid droplets, tails and antenna loss and build-up of algae and bacteria around the *Daphnia*. D) Tail loss occurs in proportion to body size at the same rate in both female (red, $n = 25$) and male (blue, $n = 25$) *D. magna* Bham 2 as a percentage of lifespan (linear regression model: combined tail loss as percent of lifespan $p = 4.20 \times 10^{-5}$, tail loss between genders $p = 0.94$, female only $R^2 = 0.66$, $p = 0.0023$; males only $R^2 = 0.90$, $p = 0.0042$). E) Fecundity declines with age in *D. magna* Bham 2. Cumulative neonate production significantly declines with age (log₂ transformed age; $n = 25$, linear regression model: $R^2 = 0.77$, $p = 2.22 \times 10^{-16}$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

is easily achievable due to the availability of various genetic manipulation tools such as CRISPR, TALEN and RNAi for *Daphnia* (Schumpert et al., 2016; Nakanishi et al., 2016; Kumagai et al., 2017). Furthermore, *Daphnia* shares the most genes in common with humans among any other arthropod and other established invertebrate model organisms of ageing (Colbourne et al., 2011). Most interestingly, genetically identical female and male *Daphnia* have evolved different average lifespans

(Schwarzenberger et al., 2014), providing a unique opportunity for understanding the underlying mechanisms of ageing and regulation of lifespan.

Sex differences are commonly observed in many biological aspects with nearly all species exhibiting conditional differences in longevity except in humans where females are known to have a ubiquitous survival advantage over males. Investigating sex differences in longevity

could provide insight into principal mechanisms of ageing and lifespan regulation. However, this area of research is extremely understudied and majority of the research on sex differences in longevity has been focused on observing wild animals in their natural environment in a bid to elucidate underlying differences in life history traits and behaviour of different genders. Although the supporting evidence is weak, these observations have formed the foundation of multiple evolutionary hypotheses regarding the origin of sex differences in longevity, such as variable vulnerability to environmental risks (Williams George, 1957), sexual selection pressures (Promislow, 1992) and parental care regimes (Allman et al., 1998) in addition to more mechanistic drivers, such as asymmetric inheritance of sex chromosomes (Trivers, 1985) and mitochondria (Tower, 2006). Results obtained from observing animals in uncontrolled environments means reliability can be unclear especially when considering factors such as risk-taking behaviour which may result in a bias towards mortality in one gender compared to the other. Given this uncertainty the current understanding of sex difference is useful for insight into causes of ageing but leaves more to be determined for mechanistic differences for sex difference and intrinsic rate of ageing (Austad and Fischer, 2016). Therefore conducting controlled experiments in model organisms that show considerable sex-dependent variation in the lifespan and ageing rate can greatly improve our understanding of the ageing process. Given the cyclical parthenogenetic nature of *Daphnia* reproduction the resultant genetically identical female and male populations provide a useful platform for investigating sex differences in longevity. Male *Daphnia magna*, although genetically identical to their mothers and sisters (Hebert and Ward, 1972), under laboratory conditions have significantly shorter lifespan compared to female *Daphnia*. This can potentially be attributed to the differences in biological roles, energy expenditure (EE) and metabolism (Manini, 2010; Plaistow et al., 2015), strategies employed to maximise fitness, rates of damage to macromolecules and ageing between females and males (Pietrzak et al., 2010a). Therefore in this study our aim was to (I) provide evidence in support of establishing genetically identical female and male *Daphnia* as unique and valuable resources for research into mechanisms of ageing and (II) to delineate the mechanism involved in sex differences in lifespan. This was achieved by establishing and investigating several biomarkers of ageing and comparing the rates of DNA damage and DNA repair capacity in genetically identical female and male *Daphnia*. Furthermore, to identify life stages that the rate of ageing alters for males and females. Our data clearly demonstrates decline in heart rate, movement, antioxidant protection and DNA repair capacity as well as increase in DNA damage and lipid peroxidation as a percentage of lifespan in both genders. Most importantly, we also establish that the rate of these changes is significantly different between the two genetically identical genders, with males displaying approximately two fold higher rate of DNA damage accumulation. Overall, our data indicates that investigating sex differences in longevity in the clonal organism *Daphnia* under controlled laboratory conditions can provide insight into principal mechanisms of ageing and lifespan regulation.

2. Results

2.1. Genetically identical female and male *Daphnia magna* have significantly different lifespans

Life history studies were undertaken for female and male *D. magna* Bham 2 strain under optimal conditions as described by Kilham et al. (1998), monitoring survival, growth, tail length, age of sexual maturity and reproduction (female only). Survivorship patterns are significantly different between the genetically identical female and male *Daphnia* (Fig. 1A; log-rank test: $p = 0.0002$, $X^2 = 56.2$, $df = 1$). The medium lifespan of female *D. magna* Bham 2 strain is twice as long as male *D. magna* Bham 2 strain, with a medium lifespan of 83 days (95% CI: 77–91, $n = 50$ individuals) and 41 days (95% CI: 38–45, $n = 50$

individuals), respectively.

Although lifespan is significantly different between the genders, as a percent of their lifespan, growth and tail loss occur at comparable times. Body length, width and tail length were monitored every 10 days from aged 1 day until death. As shown by Fig. 1B and Supplementary Fig. S2A, body size is significantly different between genders ($p = 2.2 \times 10^{-16}$) with females being approximately twice as large as males. However, body length and body width show a stabilisation of growth following 40% of lifespan in both female and male individuals (Supplementary Figs. S1 and S2B). Tail length declines with age as identified in Fig. 1C. When tail loss is normalised to body size it becomes clear that tail length is proportional to body size across both genders (Fig. 1D). Tail loss is significantly linked with age but as percent of lifespan has no significant difference between genders (linear regression model: tail loss as percent of lifespan $p = 4.20 \times 10^{-5}$, tail loss between genders $p = 0.94$).

Daphnia spp. exists as females under non-stressed conditions and reproduce via cyclical parthenogenesis. *D. magna* strain Bham 2 become sexually mature at aged 9 days when the first clutch appears in the brood pouch which are released as neonates at aged 11 days. Neonates were counted daily throughout life and cumulative neonate production was assessed (Fig. 1E). Fecundity declines with age ($p = 2.22 \times 10^{-16}$) and begins to regress early in the female life cycle at approximately aged 20 days but notably reproduction continues throughout lifespan. In nature males are produced under stressed conditions to allow the production of resting eggs by sexual reproduction but in laboratory conditions can be induced by exposure to the juvenile hormone methyl farnesoate (MF). Bham 2 males induced by MF were identified as sexually mature at aged 8 days by the development of hook-like first appendages used for clasping females during mating.

2.2. Deterioration of physiological functions in ageing female and male *D. magna*

As shown in Fig. 2A and B, although the average heart rate per minute (BPM) for males is consistently higher than the female *D. magna* Bham 2, both genders show a significant reduction (as percentage of lifespan: $p = 9.6 \times 10^{-41}$, as age in days: $p = 2.7 \times 10^{-74}$) in heart rate as they age. In females, heart rate decreases by 1.6 fold in 100 days old *Daphnia* (100% of lifespan) compared to 10 days old *Daphnia* (10% of lifespan). In males, the same trend is observed with a significant 1.2 fold reduction in the heart rate of 50 days old *Daphnia* (100% of lifespan) compared to 10 days old *Daphnia* (20% of lifespan). Most importantly, the rate of decline is significantly different between the two genders with a higher rate of decline in the short lived male *Daphnia* compared to females which is less prominent when comparing the heart rate of the two genders as percentage of their lifespan rather than their chronological age. As percentage of lifespan the BPM declines 2.02 fold faster in male *Daphnia* compared to female *Daphnia* (slopes for male and female *D. magna*, respectively are -1.196 and -0.5916 , $p = 1.4 \times 10^{-96}$). As age in days the BPM declines 4.04 fold faster in male *Daphnia* compared to female *Daphnia* (slopes for male and female *D. magna*, respectively are -2.391 and -0.5916 , $p = 3.6 \times 10^{-57}$).

The average speed of swimming, measured as the distance (cm) travelled per second, also declines progressively with age in both female and male *D. magna* (combined data analysis: $R^2 = 0.82$, $p = 1.81 \times 10^{-94}$). The average swimming speed for 80 days old female *D. magna* (80% of lifespan, 0.87 ± 0.56 cm/s) significantly reduced by 3 fold compared to 10 days old female *D. magna* (10% of lifespan, 2.63 ± 1.35 cm/s). A similar trend was observed for male *D. magna* where 40 days old males (80% of lifespan, 0.89 ± 0.23 cm/s) showed a 1.5 fold reduction in their average swimming speed compared to 10 days old males (20% of lifespan, 1.34 ± 0.38 cm/s). Furthermore, no movement was detected within 3 min of exposure to the stimulus light for both female and male *D. magna* at 100% of their lifespan (100% of lifespan equals to 100 days old and 50 days old

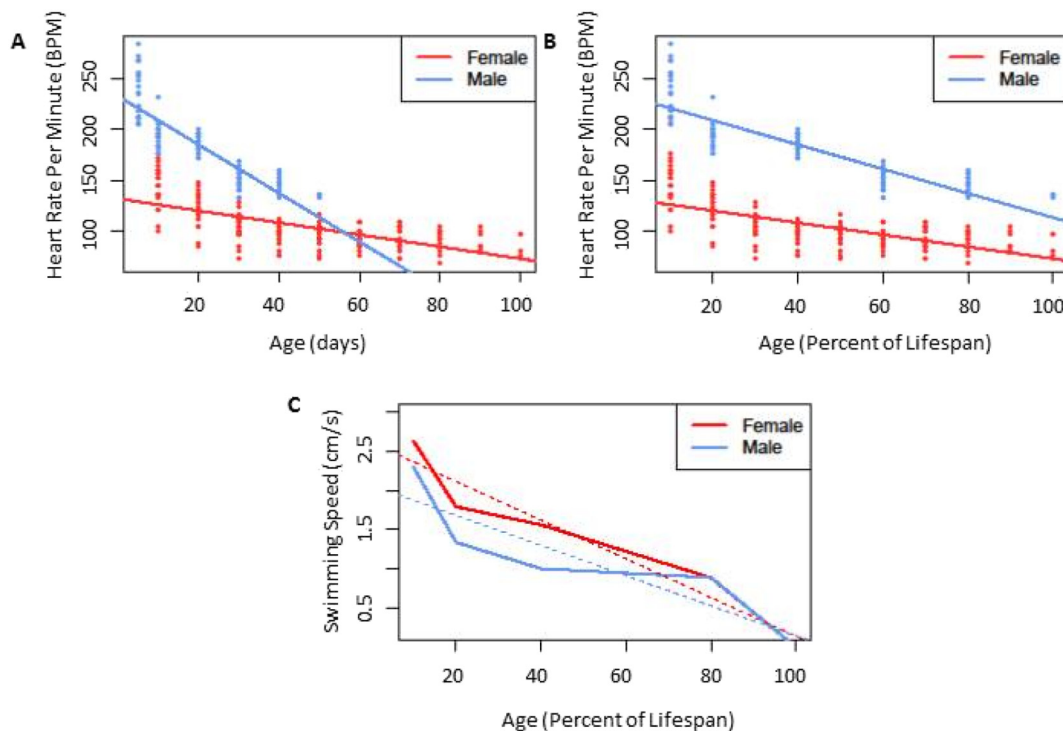


Fig. 2. Deterioration of physiological functions with age in male and female *Daphnia magna* Bham2. A) Heart rate per minutes (bpm) versus age (day) in female and male *Daphnia magna*. Heart rate decline (bpm) is occurring much earlier in males (blue, $n = 5$ biological replicates, each biological replicate containing 5 number of *Daphnia*) than females (red, $n = 5$ biological replicates, each biological replicate containing 5 number of *Daphnia*) in real time. The blue (male) and red (female) lines represent the mean linear regression (in combined analysis $R^2 = 0.81$, $p = 1.81 \times 10^{-94}$). Heart rate decline is significant with age ($p = 2.7 \times 10^{-74}$) and between the two genders ($p = 3.6 \times 10^{-57}$). Both genders show a significant decrease in heart rate with age (female only $R^2 = 0.46$, $p = 14.84 \times 10^{-24}$. Male only $R^2 = 0.75$, $p = 1.12 \times 10^{-28}$). B) Heart rate per minutes (bpm) versus percentage of lifespan in female and male *Daphnia magna*. The blue (male, $n = 5$ biological replicates, each biological replicate containing 5 number of *Daphnia*) and the red (females, $n = 5$ biological replicates, each biological replicate containing 5 number of *Daphnia*) lines represent the mean linear regression ($R^2 = 0.84$, $p = 6.32 \times 10^{-105}$). Heart rate decline is significant as a percentage of life ($p = 9.6 \times 10^{-41}$) and between the two genders ($p = 1.4 \times 10^{-96}$). Both genders show a significant decline in heart rate as percent of lifespan (female only $R^2 = 0.46$, $p = 1.84 \times 10^{-24}$, males only $R^2 = 0.75$, $p = 6.32 \times 10^{-105}$). C) Swimming speed versus percentage of lifespan for female (red, $n = 3$ biological replicates, each biological replicate containing 5 number of *Daphnia*) and male (blue, $n = 3$ biological replicates, each biological replicate containing 5 number of *Daphnia*) *Daphnia magna*. Swimming speed (cm/s) declines with age in both genders with age (R^2 for combined analysis = 0.82 , $p = 1.81 \times 10^{-94}$). Decline in swimming speed is significant in females and males (female only $R^2 = 0.93$, $p = 0.0075$, males only $R^2 = 0.81$, $p = 0.039$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

female and male *D. magna*, respectively).

2.3. Increased lipid peroxidation and decline in antioxidant protection in ageing female and male *D. magna*

A significant increase in the lipid peroxidation product free malondialdehyde (FrMDA) was identified as a function of age in both females and male *D. magna* (Fig. 3A, combined analysis for both genders: $p = 0.0004$; female: $p = 1.68 \times 10^{-07}$; male: $p = 3.28 \times 10^{-09}$). For female *D. magna* aged 20 days (20% of lifespan) the average FrMDA content (103.85 ± 37.11 nMoles MDA/mg protein) increased 4.98 fold compared to aged 100 days (100% of lifespan, 517.48 ± 39.50 nMoles MDA/mg protein). A similar trend is observed in male *D. magna* where the average FrMDA content increased 8.77 fold in 50 days (100% of lifespan, 57.37 ± 3.79 nMoles MDA/mg protein) old male *D. magna* compared to 10 days old (20% of lifespan, 6.54 ± 1.58 nMoles MDA/mg protein).

For the same samples, levels of the antioxidant glutathione (GSH) were measured and showed to be significantly declining with age in female and male *D. magna* (Fig. 3B, $p = 0.009$). Females observed an average decline of 3.25 fold between aged 20 days (20% of lifespan, 446.5 ± 53.25 nMoles GSH/mg protein) and aged 100 days (100% of lifespan, 137.26 ± 17.17 nMoles GSH/mg protein). Males also showed an average decline of 3.18 fold between aged 10 days (20% of lifespan, 622.31 ± 50.89 nMoles GSH/mg protein) and aged 50 days (100% of

lifespan, 195.48 ± 109.05 nMoles GSH/mg protein). The observed increase in peroxidation product FrMDA and decline in the protective antioxidant GSH suggests a more vulnerable environment to oxidative stress.

2.4. DNA damage accumulation and reduced repair capacity with age in female and male *Daphnia*

Comet assay was used to calculate DNA damage by measuring tail intensity (%). The more damaged and fragmented the DNA the larger the percentage of DNA found in the comet tail. A clear accumulation of DNA damage can be seen with age in both female and male *D. magna*. When presented as percent of lifespan damage appears to accumulate at a similar rate in both genders initially before diverging to show higher damage in females from 60% lifespan compared to males (Fig. 4A, significance between genders $p = 1.70 \times 10^{-05}$) but when presented in real time (chronological age) it is clear that DNA damage is accumulating significantly faster in males (Fig. 4B, significance between genders $p = 1.80 \times 10^{-23}$, 1.49 fold higher rate of DNA damage accumulation in male *D. magna* than female *D. magna*). When investigating genders individually it is clear that DNA damage accumulates significantly with age in both females and males (female $R^2 = 0.48$, $p = 3.30 \times 10^{-87}$; male $R^2 = 0.35$, $p = 1.40 \times 10^{-35}$).

To investigate the efficiency of DNA damage repair throughout age, female and male *D. magna* were exposed to $0.1 \mu\text{g/mL}$ cisplatin and

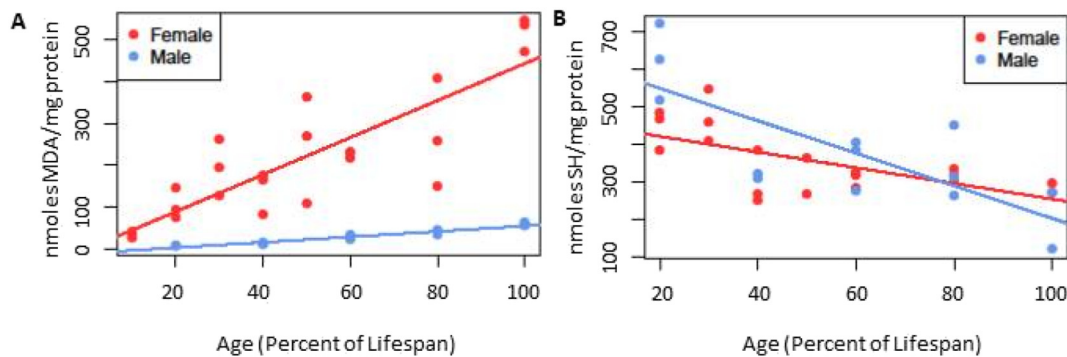


Fig. 3. Oxidative stress increases with age in female and male *D. magna*. A) An increase in the lipid peroxidation product free malondialdehyde (FrMDA) is observed with age in female (red) and male (blue) *D. magna*. The rate of FrMDA accumulation with age is significantly different between genders ($p = 2.00 \times 10^{-08}$) but a significant increasing trend is apparent in both genders (combined linear regression model: as percent of lifespan + gender $R^2 = 0.65$, $p = 6.38 \times 10^{-09}$, percent of lifespan only $p = 3.90 \times 10^{-04}$). Lipid peroxidation product accumulation with age is significant in both female and male individuals (female only $R^2 = 0.72$, $p = 1.68 \times 10^{-07}$; male only $R^2 = 0.94$, $p = 3.28 \times 10^{-09}$). B) A decline in thiol content is evident in female (red) and male (blue) *D. magna* (combined linear regression model: as percent of lifespan + gender $R^2 = 0.42$, $p = 6.38 \times 10^{-05}$, percent of lifespan only $p = 9.10 \times 10^{-03}$, gender only $p = 1.50 \times 10^{-04}$). Decline in reduced thiols is significant in both genders separately (female only $R^2 = 0.41$, $p = 0.0033$; males only $R^2 = 0.59$, $p = 0.0022$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

damage (γH2AX) and repair (RAD51) markers were monitored at specified recovery stages (0, 1.5, 3 and 6 h of recovery) for the exposure groups and compared to control groups. Western blot analysis of the RAD51 confirmed specificity of the antibody used (Supplementary Fig. S3). As previously discussed DNA damage significantly increases with age including specifically an increase in double strand breaks (DSBs) identified by a 4.16 fold increase in average γH2AX foci count ($p = 0.000$) between control females aged 80 days (5.82 foci) and control females aged 10 days (1.40 foci) (Fig. 4C, Supplementary Fig. S4). A sharp increase in γH2AX foci following cisplatin exposure can be seen across all age groups however a distinct difference in damage throughout recovery is notable between younger ages compared to aged 80 day females (Figs. 4C and 5, see Supplementary Table S1 for significance values). By 6 h of recovery females aged 10 days have significantly higher recovery than older individuals identified by an average γH2AX foci count 18.83 fold lower compared to females aged 80 days (0.41 and 7.72 respectively, $p = 0.039$). As seen in Fig. 4D, repair foci also show a distinct difference between young and old females whereby at 6 h of recovery RAD51 foci count for aged 10 days has 1.43 fold higher count compared to aged 80 days (7.41 and 5.17 respectively, $p = 0.046$). Also of note is the little variation between control RAD51 foci count and all recovery stages in females aged 80 days showing a lack of repair response to cisplatin exposure (multi-comparison post-hoc test resulted in a range of p -values between age groups ranging between 0.319 and 0.999, no significant difference between control and all recovery groups for females aged 80 days, see Supplementary Table S1). In male samples as shown in Fig. 4E and F, at 6 h of recovery γH2AX foci count is 1.59 fold higher ($p = 0.002$) at aged 40 days (7.63) compared to aged 10 days (4.80). RAD51 expression increases in all age groups following cisplatin exposure however younger males appear to respond better with a maximum RAD51 foci count for aged 10 days of 8.21 whilst maximum foci count for aged 40 day males is 7.04. The clear decline in damage in younger *D. magna* in addition to high repair foci count compared to the continually high damage foci and unresponsive repair foci in older *D. magna* suggests repair efficiency is diminished with age.

3. Discussion

In this study we examined biomarkers of ageing in genetically identical female and male *Daphnia magna* with diverse lifespans to support their establishment as a unique and valuable model for investigating sex difference in longevity. Such work is necessary to elucidate principal mechanisms of ageing and lifespan regulation.

3.1. Sex differences in physiological markers of health and ageing in *D. magna*

Physiological markers were observed in the form of life history studies. Life history studies form an analytical platform to investigate strategies adopted by an organism via natural selection covering traits such as lifespan, growth, age of sexual maturity and fecundity. Monitoring the age and stage-specific patterns of an organisms' life offer insight into physical and ecological dependencies of the organism. Given the predominant existence of female *D. magna* during non-stressed conditions, life history data for females provide baseline data which offer a reference to compare other conditions against. When establishing an ageing model this baseline data is essential for identifying key variations in modified conditions and in this instance highlight key differences between female and male *D. magna* in real-time, most importantly a significant difference in lifespan, but show as a percent of lifespan both are experiencing comparable life history traits.

A widely observed gender difference aside from lifespan is body size. Commonly referred to as sexual size dimorphism, the observed difference in size between adult females and males of a population (in both animals and plants) is often related to separate roles in reproduction (Fairbairn et al., 2007). As shown in Fig. 1B, female *D. magna* are approximately twice as large as their male counterparts. As naturally occurring populations of *D. magna* consist of females that reproduce via cyclical parthenogenesis to produce clonal daughters, it is suggested that females have adopted faster growth rate and larger body size to maximise energy availability for reproduction of multiple broods. Such adoptions maximise the number of produced offspring and promotes population survival (Pietrzak et al., 2010a). Furthermore, male *D. magna* become established under non-favourable conditions which force the female *D. magna* to switch reproductive strategy to become sexually receptive to males. It is under stressed conditions that males are required for the production of 'resting eggs'. Resting eggs are encased in a hard outer capsule known as ephippia which allows the eggs to lay dormant in sediment until more favourable conditions return (Hebert, 1987). Given the biological role of males is for sexual reproduction in unfavourable conditions, different mechanisms have evolved to maximise male fitness. For example, here we see the males are much smaller and do not exert excess energy in a fast growth rate and large body size as seen in females, but instead may direct energy in to maximising egg fertilisation which would require contact with as many females as possible (Pietrzak et al., 2010a). Also notable is the similarity of age at sexual maturation in females and males, aged 9 days and 8 days respectively. It is possible that the shorter lifespan, smaller

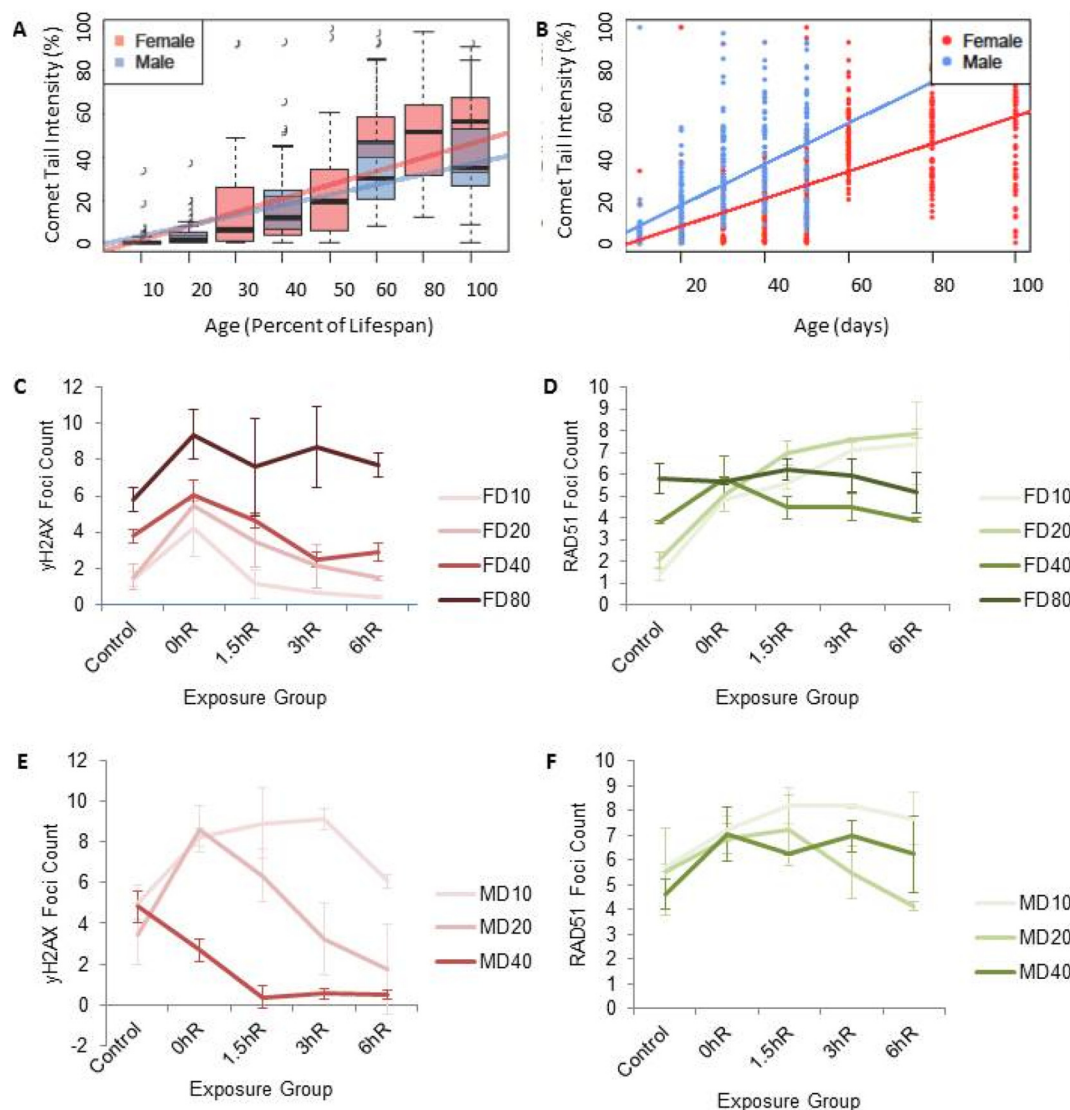


Fig. 4. Accumulation of DNA damage and reduced repair capacity with age in female and male *D. magna*. A) Comet assay identifies accumulated damage in female and male *D. magna* as a percent of lifespan (combined linear regression model: as percent of lifespan and gender: $R^2 = 0.43$, $p = 2.32 \times 10^{-118}$, percent of lifespan only $p = 2.00 \times 10^{-117}$, gender only $p = 1.70 \times 10^{-05}$; females: $n = 3$ biological replicates, each replicate containing haemolymph from 5 Daphnia, total number of 75 nuclei scored; males: $n = 3$ biological replicates, each replicate containing haemolymph from 5 Daphnia, total number of 75 nuclei scored). B) Comet assay analysis presented as age in days shows higher rate of accumulation of damage in male *D. magna* compared to females (combined linear regression model: age in days $p = 3.30 \times 10^{-105}$, gender $p = 1.80 \times 10^{-23}$). C) Quantification of yH2AX foci in female *D. magna* for control and cisplatin exposed haemolymph show higher foci count in older individuals (dark red) compared to younger individuals (light red). Error bars: SD; females: $n = 3$ biological replicates, each replicate containing haemolymph from 10 female Daphnia, 75 number of foci scored. D) Quantification of RAD51 foci in female *D. magna* for control and cisplatin exposed haemolymph show an increased response to damage in younger individuals (light green) compared to older individuals (dark green). Error bars: SD. females: $n = 3$ biological replicates, each replicate containing haemolymph from 10 female Daphnia, 75 number of foci scored. E) Quantification of yH2AX foci in male *D. magna* for control and cisplatin exposed haemolymph show higher foci count in older individuals (dark red) compared to younger individuals (light red). Error bars: SD; males: $n = 3$ biological replicates, each replicate containing haemolymph from 10 male Daphnia, 75 number of foci scored. F) Quantification of RAD51 foci in male *D. magna* for control and cisplatin exposed haemolymph show an increased response to damage in younger individuals (light green) compared to older individuals (dark green). Error bars: SD. males: $n = 3$ biological replicates, each replicate containing haemolymph from 10 male Daphnia, 75 number of foci scored. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

size and age at maturation of male *D. magna* is due to the biological role males play in reproduction.

In cases of sexual size dimorphism a divergence of life history and ecological variables is often observed (Fairbairn et al., 2007). Although body size differs between genders, it is observed that both females and males reach maximum growth at approximately 40% of their lifespan. In a similar fashion, relative to body size both genders lose their tail at a similar rate as percent of lifespan (Fig. 1D) however when considering respective lifespans this means both maximised growth and rate of tail loss is occurring twice as fast in real time in males compared to females.

Across many species that undergo appendage loss and regeneration it is often observed that the regenerated appendage is smaller in comparison to non-regenerated appendages. Notably these smaller appendages can result in a negative impact on foraging, reproduction and/or survivorship. Furthermore, regeneration capacity declines with each instar therefore as the animal ages regenerative ability is reduced (Maginnis, 2006).

As shown by Fig. 2C, females appear to swim faster than males which may be an evolutionary divergence necessary for maximal survival. In nature, females are likely to frequent closer to the surface of

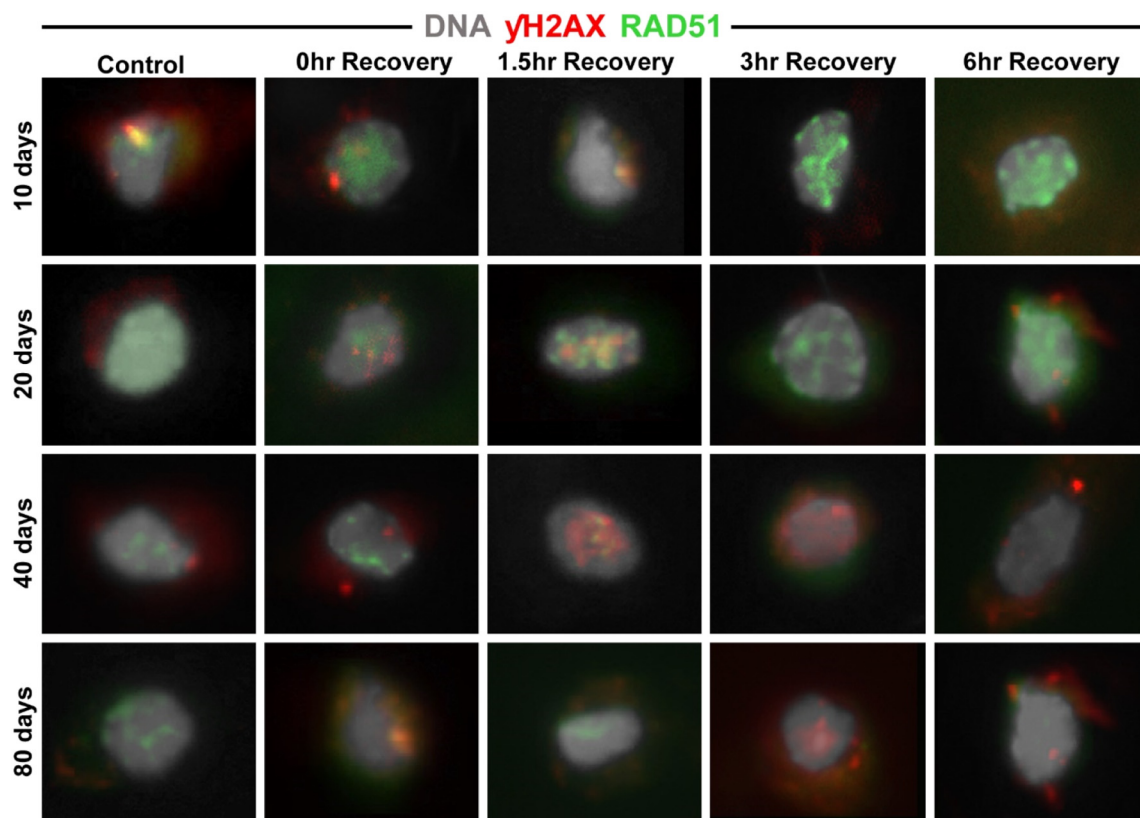


Fig. 5. Representative images of γ H2AX and RAD51 foci in female *D. magna*. Panel images representative of γ H2AX (red), RAD51 (green) and Hoechst (blue) staining in female *D. magna* for untreated control groups, and at recovery time points following 6 h cisplatin exposure for age groups 10, 20, 40 and 80 days. Foci highlighted by colour for γ H2AX representing damage (red) and RAD51 representing recovery (green). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the waterbody where food is more plentiful to aid maximal reproduction and growth. However, higher risk of predation is also present closer to the surface therefore a faster swimming speed may be essential for survival. Additionally, males are often situated further away from the surface in cooler conditions with less risk of predation (Pietrzak et al., 2010a). It is believed the response of *Daphnia* to swim vertically away from light (diel vertical migration) has evolved for two reasons; firstly, to minimise mortality via predation by descending to darker depths of the waterbody, and secondly, to maximise development and reproductive rates by locating near the warmer surface of the waterbody throughout the night (Dodson et al., 1997). Irrespective of speed, both genders express a significant consistent decline in swimming ability with age. *Daphnia* swim using the second set of antennae which are controlled by large muscles. The reduction in swimming speed may be potentially linked to age-dependent loss of strength and mass in this muscle however very little more is known about this mechanism. Furthermore, we also observed an age-dependent decline in heart rate in both genders of *D. magna* which may be linked to reduced swimming speed (0.0092). Similar observations have been recorded in *Drosophila melanogaster* where heart rate decline and reduced climbing ability have been linked to the observation in humans where a reduced resting heart rate is observed with age in addition to a more pronounced decline in maximum heart rate during exercise (Paternostro et al., 2001). It is possible that *Daphnia* experience an age-dependent increase in muscle frailty as seen in other species which is linked to a time-dependent loss of muscle mass, integrity and function (Demontis et al., 2013), which if it is true, is occurring at different rates here between female and male *D. magna*. Additionally, *Daphnia* unlike most other invertebrates have a myogenic heart (Baylor, 1942) which may be utilised to investigate cardiac muscle degeneration and heart rate decline with age in addition to gender differences. These prospects should be investigated further.

Additionally, the inverse relationship between heart rate and lifespan presented here has also been identified across many species including humans (Gent et al., 2015) which has been linked to multiple factors including protein oxidation, free radical production, inflammation and telomere shortening (Zhang and Zhang, 2009). Interventions used to slow heart rate have resulted in extended lifespan (Singh, 2001; Cook et al., 2006; Gent et al., 2015) further supporting the role of heart rate on maximum lifespan. Effect of heart rate on longevity is also observed within species, evidenced by comparing lifespan of hibernating and non-hibernating bats. During hibernation heart rate is significantly slowed and it is observed that these hibernating bats live 70% longer than their non-hibernating counterparts (Cook et al., 2006). Additionally, body mass has been associated with heart rate and longevity in mammals, notably smaller mammals tend to show higher heart rate and shorter lifespan (Zhang and Zhang, 2009). Male *Daphnia magna* exhibit a much higher heart rate and a much smaller body mass therefore it is possible that this contributes to the observed lifespan of male *Daphnia magna* being approximately half that of their female counterparts. Due to the higher heart rate and shorter lifespan observed in males it is possible that the age-dependent decline in heart rate is occurring at a much faster rate in males compared to females.

As previously described, we see a decline in overall swimming speed with age, however, also of note is the observed 'dip' in swimming speed at 20% of lifespan for females and 40% of lifespan for males, both of which equate to aged 20 days in real time. This decline in swimming speed in both genders at the day 20 time point may be linked to the observed decline in reproductive capacity expressed by females at the same age (Supplementary Fig. S5). Initial reproduction between aged 11 and 20 days shows a steep increase however from aged 20 days reproduction begins to decline. The decline in female swimming speed

may be attributed to the lesser demand for feeding to produce large broods whilst the males may be linked to age-dependent changes in mating behaviour and the potentially reduced fertility of the females after this time. It has been recorded in humans that both female and male fertility declines with age. Males can experience what is termed as 'male factor infertility' which encompasses various conditions related to sperm count, mobility and morphology (Harris et al., 2011). The observation of female and male fertility decline with age may be applied to *D. magna* and the observed decline in swimming speed may be as a result of reduced mating potential.

The gradual decline in reproduction with age seen in female *D. magna* (Fig. 1E) is a trait often observed in humans whilst a complete stop in reproduction after a certain age is observed in *C. elegans* and *Drosophila* (Grotewiel et al., 2005; Kenyon, 2010). It is widely known that as *Daphnia* grow they have a higher demand for food. Feeding a fixed volume throughout lifespan could therefore lead to limitation of growth if not enough food is present, which in turn would result in modified resource allocation and thus lead to decline in reproduction. However, in a controlled laboratory environment it was clear that algae was present in the media at the time of media change suggesting an excess of algae was present therefore food is unlikely to have been a limiting factor. Female *D. magna* produce eggs from nurse cells in the ovary continuously throughout their lifecycle but the number of offspring per brood declines and time between broods increases with age. The observed decline in reproduction may be in part due to the impaired stem-cell function with age as observed in both vertebrates and invertebrates. One such contributing factor to this impairment is DNA damage indicated by comet analysis and higher levels of γ H2AX and impaired DNA repair identified in ageing mice hematopoietic stem cells (Ahmed et al., 2017). The ability of *D. magna* to utilise nurse cells throughout life makes them a potentially interesting tool for stem cell research.

3.2. Sex differences in molecular markers of health and ageing in *D. magna*

Ageing is not only observed through physiological decline but also encompasses molecular and cellular changes. Here we demonstrate the decline in reduced glutathione (GSH) in addition to increased lipid peroxidation product malondialdehyde (MDA) with age (Fig. 3A and B). One of the most well supported theories of ageing is the DNA damage theory (Gensler and Bernstein, 1981). This theory suggests the observed age-associated functional decline is largely caused by DNA damage accumulation over time which leads to cellular alterations and tissue homeostasis disruption (Freitas and de Magalhães, 2011). However, the mechanisms driving genomic instability are only recently starting to be uncovered. One such mechanism suggested is an increase in macromolecule peroxidation which may be driven by depletion of antioxidant defence mechanisms such as thiol-containing glutathione. GSH works as a protective barrier against oxidative damage by acting as a substrate for glutathione peroxidases including hydrogen peroxide and lipid peroxide. When oxidised, GSH takes on a disulphide form (GSSH). The age-related shift observed between GSH/GSSH redox couples has been identified in clinical studies in humans and is thought to contribute to ageing diseases such as cardiovascular disease, diabetes, rheumatoid arthritis and neurological disorders (Giustarini et al., 2006), similar to observations in *Drosophila* where GSH/GSSG ratios significantly decrease as the flies age (Rebrin et al., 2004). It has been suggested that the age-related decline in GSH is partly driven by an increased oxidative load over time (Giustarini et al., 2006). Our results support the current understanding of GSH depletion and increased oxidative insult with age which suggests lesser protection and higher damaging load may be contributing to increased DNA damage with age. Notably, male *D. magna* appear to have consistently lower amounts of MDA compared to females when normalised to protein content. Lipid peroxidase preferentially oxidises poly-unsaturated fatty acids (Gaschler and Stockwell, 2017) therefore it is possible this difference between genders

is linked to differences in lipid composition which may exist due to variable reproductive demands.

Consistent with the DNA damage theory of ageing, here we demonstrate both female and male *D. magna* experience DNA damage accumulation with age at comparative rates as a percentage of their lifespan (Fig. 4A). However, in real time DNA damage is accumulating at a much faster rate in males compared to females (Fig. 4B). The reported DNA damage accumulation may be in part due to the previously described decline in antioxidant protection from increasing peroxidation, but may also be a result of age-related decline in DNA damage repair efficiency. Using the direct marker of DNA damage γ H2AX (Sharma et al., 2012) we identified an increase in DNA damage with age indicated by higher γ H2AX foci in ageing control groups (Fig. 4C) supporting the accumulation of naturally occurring DNA damage shown by comet analysis. Concurrently, we see a similar pattern with RAD51 with higher expression of RAD51 apparent in older *D. magna* (Fig. 4D). To monitor change in repair efficiency with age both female and male *D. magna* were exposed to cisplatin. Cisplatin is a DNA damaging agent that works by 3 different mechanisms; A) addition of alkyl groups to DNA bases which leads to repair enzymes fragmenting the DNA when trying to substitute the alkylated base, B) by forming cross-links which inhibits synthesis or transcription and C) inducing mutations by mispairing nucleotides (Dasari and Tchounwou, 2014). Following cisplatin exposure a clear increase in γ H2AX foci is observed across all female age groups (Fig. 4C) as expected, however it becomes apparent that when in recovery and no longer exposed to cisplatin younger animals revert back to control damage levels (aged 10 days by 1.5 h, aged 20 days by 6 h, aged 40 days between 3 and 6 h) whereas females aged 80 days do not recover in the 6 h window. Similar findings are observed in *Drosophila melanogaster* whereby γ H2AX foci persist for longer in older spermatogonia (Delabaere et al., 2017). This gradual reduction in recovery may be in part due to the decline in efficiency of repair via homologous recombination, a preferred repair pathway for double strand breaks. RAD51 plays an essential role in homologous recombination by driving recognition of homology and strand exchange between homologous templates. Following cisplatin exposure, younger *D. magna* show an increase in RAD51 foci count and at ages 10 and 20 days levels of RAD51 remain high. By aged 80 days there is no significant difference between control levels of RAD51 compared to all recovery stages. This may suggest that repair via homologous recombination is at its maximum capacity in older animals due to the higher naturally occurring damage present with old age. Another possibility is shared by Delabaere et al. (2017) which proposes that homologous recombination becomes defective with age by the inability of RAD51 to dissociate. If RAD51 does not dissociate it blocks the progression of effective repair resulting in persisting damage which may explain not only the higher baseline levels of γ H2AX and RAD51 foci in older animals but would also inhibit recovery of older *D. magna* exposed to the DNA damaging agent cisplatin.

Male *D. magna* aged 10 days follow a similar trend to female *D. magna* in terms of increased γ H2AX foci immediately after cisplatin exposure followed by a decline during the recovery period whilst RAD51 remains high for the duration. However, control unexposed males appear to express higher levels of γ H2AX and RAD51 even at a younger age comparatively to females of the same age and similar percent of lifespan (Supplementary Fig. S6a and b). Control males aged 10 days have an average γ H2AX foci count of 3.46 and RAD51 foci count of 5.68 which is much higher than females aged 10 days with 1.40 and 1.42 respectively, or an equivalent of 20% lifespan at females aged 20 days showing an average foci count of 1.53 and 2.07 respectively. Given the maximum recorded average for both γ H2AX and RAD51 foci is approximately 9 foci it would appear that male individuals are closer to maximum damage and recovery capacity much earlier in their lifetime. As the males age the trend of γ H2AX and RAD51 foci begins to differ to that seen in the females. RAD51 is not only integral to DNA repair, but also in cell cycle arrest at the G2/M

checkpoint where DNA damage is repaired prior to mitosis. A defective G2/M checkpoint results in death after cell division (Kostyrko et al., 2015). Given the dysregulation of RAD51 in DNA repair with age (Delabaere et al., 2017) it is possible that the G2/M checkpoint is also compromised given the importance of RAD51 signalling in the progression in to the G2 phase (Kostyrko et al., 2015). Furthermore, γ H2AX is an important molecule in the decision of a cell to continue to DNA repair or apoptosis. Higher expression of γ H2AX (with dual phosphorylation at Ser139 and Tyr142) promotes a pro-apoptotic conclusion (Cook et al., 2009). It is possible that cisplatin exposure in older males causes severe unrecoverable damage promoting apoptosis which results in the differing trend we see in male γ H2AX foci compared to females.

Overall, our data clearly shows that lifespan and ageing rate can vary in genetically identical genders of one specie. Physiological and molecular markers of ageing measured in this paper demonstrate that age-associated changes are occurring at the same rate as percentage of lifespan. However considering that males have half the lifespan of females this indicates that the age-associated changes are occurring approximately twice as fast in male *D. magna* compared to females. The data presented in this paper supports the use of this unique model organism for research into sex differences in longevity and ageing. They provide a suitable platform for investigating the contribution of non-genetic factors and mechanisms, such as epigenetic factors, hormones, environmental factors, energy allocation and trait offs in determining lifespan and ageing rate.

4. Experimental procedures

4.1. *Daphnia magna* stocks and maintenance

Cultures of *Daphnia magna* Bham 2 strain (originally obtained from the University of Reading (Heckmann et al., 2006) have been maintained in the laboratory conditions at the *Daphnia* Facility, University of Birmingham, UK for over 10 years as previously described (Athanasio et al., 2016; Athanasio et al., 2018; Kilham et al., 1998). Briefly, *D. magna* Bham 2 strain were maintained in photoperiodic lighting (16 h of light:8 h of dark) and temperature of $20 \pm 1^\circ\text{C}$, in high hardness COMBO medium (HH COMBO). Animals were fed every other day with *Chlorella vulgaris* at a concentration of $\approx 27,550$ cells of algae per individual *Daphnia*. Media was changed three times a week and cultures maintained at a maximum of 1 adult/50 mL of media at a minimum density of 5 individuals/250 mL.

4.2. Chemical-induction of genetically identical male *D. magna* BHAM 2

A stock solution of the crustacean reproductive hormone, (E,E) Methyl Farnesoate (MF; Tebu-Bio Ltd, Peterborough, UK) was prepared by dissolving the chemical in dimethyl sulfoxide (80 mM stock concentration). The stock was stored at -80°C until use. The stock was diluted 50-fold to $1600\ \mu\text{M}$ then directly added to 50 mL of culture water (Final concentration: 400 nM) containing sexually matured individual female *Daphnia* (age of maturity for *D. magna* Bham 2 strain: 9 days old). This concentration is sufficient to induce male *Daphnia* at 100% efficiency (Olmstead and Leblanc, 2002). Due to the instability of MF, media was changed daily to ensure consistent exposure. The first brood was discarded and male neonates were collected from 2nd–5th broods. Female and male cultures were maintained separately.

4.3. Life history study

Life history studies were conducted for both female and male *D. magna* Bham 2 strain under normal culturing conditions. Survival and reproduction rate (only in females) were assessed for 50 individuals. Growth rate was calculated by measuring body length (L), body width (W) and tail length (T; $n = 25$ individuals) as described previously

(Martínez-Jerónimo, 2011; Athanasio et al., 2018). Briefly, to measure growth rate, *Daphnia* ($n = 25$ individuals) were photographed throughout their life at 10 day intervals using a stereomicroscope SMZ800 (Nikon, Japan) coupled to a digital camera DS-Fi2 (Nikon, Japan) and images were analysed using ImageJ software. Furthermore, heart rate (beats per minute; BPM) was measured for 25 individuals at 10 day intervals starting when the *Daphnia* were 10 days old until the end of their lifespan. To measure heart rate, video footages of the *Daphnia* heart were captured using NIS-Elements F imaging software and Video Velocity software.

4.4. Measuring swimming speed in different age groups of *Daphnia magna* Bham 2 strain

The method described by Whitman and Miller (1982) with some modifications, was used to measure the time required for different age groups of female (10, 20, 40, 80 days old, $n = 5$) and male (10, 20, 40 days old, $n = 5$) *Daphnia* to travel a defined distance (s/cm). The method takes advantage of the phototactic behaviour of the *Daphnia* to guide them to swim a defined route and distance away from the light source. This was achieved by placing individual *D. magna* of appropriate ages in a stripette levelled and sealed with parafilm at one end and containing the culturing media. The sealed end of the stripette was fixed 4.5 cm from the TransLight Illuminator light box (Orsam 797–3453, 8 kWh/1000 h). The *Daphnia* were allowed to acclimatise to darkness for 10 min before being exposed to light. Time taken for each *Daphnia* to travel 10 cm following exposure to light was recorded at $0.5\times$ speed using Slo Mo Video mobile application.

4.5. Comet assay

Comet assay was performed to assess accumulation of DNA damage with age in female and male *Daphnia* according to the method developed by Pellegrini et al. (2014) for *Daphnia magna*. Due to variance in DNA damage across different tissue types, haemolymph was selected as the tissue for analysis. To avoid contamination of the haemolymph with non-haemolymph cell the method described by Auld et al. (2010) was used to directly extract haemolymph from the heart of the *Daphnia*. This was achieved by piercing the haemocoel and allowing the heart to force haemolymph through the tear and directly into the collecting buffer, Phosphate Buffer Saline (PBS). Haemolymph from 5 *Daphnia* were combined per replicate for each age group ($n = 3$). The alkaline comet assay was completed following the methods in Pellegrini et al. (2014). Briefly, the single haemolymph cells were suspended in low melting point agarose (1%) then spread on slides coated with high melting point agarose (1%) and covered with a coverslip and allowed to solidify at 4°C . After solidification a second layer of low melting point agarose was added and the plasma and the nuclear membranes were digested using a lysis buffer. DNA unwinding and electrophoresis ($0.78\ \text{V/cm}$, 300 mA) were performed in an alkaline buffer (1 mM Na2EDTA, 300 mM NaOH, $\text{pH} > 13$). After electrophoresis, each slide was neutralized with 2 ml of neutralization buffer (0.4 M Tris-HCl, $\text{pH} 7.5$), fixed in 100% ethanol at -20°C , air dried and stored in closed microscope slide container before staining with SybrGold (ThermoFisher, S11494) and counting using a Zeiss Axiovert 10 microscope. Slides were scored counting 50 nuclei per sample using an automatic image analysis system (Comet Assay IV; Perceptive Instruments Limited, Haverhill, Suffolk, UK). We chose Tail Intensity (TI, %) as representative DNA damage parameter as measured by Comet Assay IV software. TI (%) is defined as the ratio of total tail intensity to total intensity of the comet expressed as a percentage (Vojnovic et al., 2019).

4.6. Immunofluorescence microscopy

4.6.1. Experimental design

Two groups of samples were used for Immunofluorescence

microscopy: (I) Female and male *D. magna* Bham 2 strain cultured under normal laboratory conditions, representing natural ageing group, (II) Female and male *D. magna* Bham 2 strain treated with cisplatin for 6 h (0.1 µg/mL dissolved in culture media, Sigma, UK), representing the group with induced DNA damage. For both natural ageing (I) and induced damaged (II) groups the following female and male ages were used, respectively: 10, 20, 40 and 80 days old females (representing 10%, 20%, 40% and 80% of the lifespan of a female *D. magna* Bham 2 strain, respectively; $n = 3$ with each replicate containing 7 individuals) and 10, 20 and 40 days old males (representing 20%, 40% and 80% of the lifespan of a male *D. magna* Bham 2 strain, respectively; $n = 3$ with each replicate containing 7 individuals). In addition, for the induced damaged group (II) not only samples were collected immediately after 6 h of cisplatin exposure (no recovery) but also samples were collected after 1.5, 3 and 6 h of recovery in clean media free of cisplatin. The later was used to assess the change in DNA damage levels and DNA repair efficiency with age.

4.6.2. Procedure

We used the immunolabelling technique described for *D. magna* by Gómez et al. (2016). Briefly, haemolymph was extracted as explained earlier and immediately added to fixative (PBS, 5% formaldehyde, 0.05% triton-X-100). The fixed haemolymph cells were transferred on to a poly-L-lysine coated slide and fixed in liquid nitrogen. Slides were washed 3×5 min in PBS followed by incubation with primary antibodies γ H2AX (1:700, Millipore 05-636) and RAD51 (1:50, Calbiochem PC130-100UL) overnight at 4 °C protected from light. Slides were washed 3×5 min in PBS before incubation with secondary antibodies goat anti-mouse (1:200, Abcam ab150115) and goat anti-rabbit (1:200, Abcam ab150078) at room temperature for 45 min protected from light. Slides were washed 2×5 min PBS and incubated with Hoechst (10 µg/µL, Thermo Scientific 62249) for 10 min at room temperature protected from light. Slides were washed 3×5 min in PBS and mounted in sterile glycerol. Slides were imaged using a Nikon Eclipse E600 microscope equipped with epifluorescence optics, a motorized z-drive, and a Hamamatsu ORCA-ER C4742-95 digital camera controlled by NIS-Elements AR v4.13 software on. A minimum of 25 foci were counted per replicate using ImageJ software. Panel images were produced using Fiji software.

4.7. Western blot

To determine the specific immunoreactivity of anti- γ H2AX and anti-RAD51, we performed Western Blot analysis of *Daphnia* haemolymph extracts. *Daphnia* haemolymph was transferred into RIPA buffer according to previous Western Blot protocols for *Daphnia* (Schumpert et al., 2016). The blot was incubated with the correspondent antibodies at a dilution of 1:1000 each, followed by incubation with HRP-conjugated goat anti-rabbit (1:200, Abcam ab150078) and goat anti-mouse (1:200, Abcam ab150115) IgG at a dilution of 1:2000. Visualization was performed using alkaline phosphatase detection system (BioRad).

4.8. Quantification of protein levels

Protein content was determined using the Coomassie Brilliant Blue G-250-based assay (Grintzalis et al., 2015). Briefly, BSA was used to create a standard curve. Female and male samples were collected at 10 day intervals between ages 10–100 days for females and 10–50 days for males (5 individuals per replicate, $n = 3$). Eggs were removed from the female brood pouch. Samples were homogenised in 800 µL deionised water using a Precellys 24 lysis and homogenisation machine (2×10 s, 2500 rpm). Bradford reagent was prepared as follows; Coomassie brilliant blue dissolved in 2 M hydrochloric acid (60 mg/100 mL), 2 M hydrochloric acid and deionised water (dH₂O) combined in a ratio of 1:1:3 respectively. Samples of each sample group were set up in a clear flat-bottomed 96-well plate; $3 \times$ blank comprising 125 µL

dH₂O plus 125 µL Bradford reagent and 4×25 µL sample, 100 µL dH₂O and 125 µL Bradford reagent. The plate was incubated at room temperature for 10 min before absorbance was read on a spectrophotometer at wavelength 595 nm. The resulting absorbance was converted to protein (µg/mL).

4.9. Quantification of the lipid peroxidation product

Lipid peroxidation products free-malondialdehyde (FrMDA) and protein-bound malondialdehyde (PrMDA) were measured following the Thiobarbituric Acid Reactive Substances (TBARS) assay (Grintzalis et al., 2013). Briefly, MDA was used to create a standard curve. The same samples used for protein quantification were combined with 25 mg/mL thiobarbituric acid (TBA) dissolved in 0.2 M sodium hydroxide and 1 g/mL trichloroacetic acid (TCA) dissolved in 12 M hydrochloric acid (HCl) in a 5:1 (sample: TBA-TCA-HCl) ratio. For reagent blanks dH₂O was used in place of the sample. Replicates were incubated for 20 min at 100 °C in a water bath with the lid of the microcentrifuge tube open. Following incubation 100% ethanol was added in a 1:1 ratio. Samples were vortexed and centrifuged to clear debris before transferring supernatant in to a black 96-well plate. Fluorescence was measured at Ex/Em 485/535 nm. Results were normalised to protein level.

4.10. Quantification of protein thiols

Concentration of reduced thiols was determined using the 4',4'-di-thiopyridine (DTP) assay (Grintzalis et al., 2014). Briefly, a standard curve was produced using glutathionine. For samples, to a clear 96-well plate 150 µL sample used for protein determination and TBARS assay was combined with 50 µL 12 M formamide and 50 mM acetic acid (pH 4.5). For the sample, 50 µL 0.75 mM DTP in 12 M formamide and 50 mM acetic acid (pH 4.5) was added but for sample blank only 12 M formamide and 50 mM acetic acid (pH 4.5) was added. For reagent blank 200 µL of 12 M formamide and 50 mM acetic acid (pH 4.5) was combined with 50 µL 0.75 mM DTP in 12 M formamide and 50 mM acetic acid (pH 4.5). Sample reagent blank comprised of only 250 µL 12 M formamide and 50 mM acetic acid (pH 4.5). Replicates were incubated at room temperature for 10 min before absorbance was measured at 325 nm.

4.11. Data analysis

Statistical analysis was undertaken using IBM SPSS Statistics 25 software package and R (v3.4.3). Data was visualised using R (v3.4.3) and Microsoft Excel 2016. Briefly, normality was tested using Shapiro-wilk test. Survival analysis was performed using the R Survival package which stipulates the use of Kaplan-Meier estimate and log-rank test. Growth data was analysed using a linear regression model testing Log₁₀ transformed age in days against measurement (mm) and as percent of lifespan using a one-way ANOVA with multiple pairwise comparison corrected by Bonferroni to accommodate for normality violation and multiple hypothesis testing. Fecundity was analysed using linear regression model testing Log₂ transformed age in days against cumulative neonate production. Average heart rate was analysed for both age in days and age as percent of lifespan using a linear regression model and using a one-way ANOVA with multiple pairwise comparison corrected by Bonferroni. Swimming speed, lipid peroxidation product and thiol content were analysed using a linear regression model. Comet tail length was analysed using linear regression model. RAD51 and γ H2AX foci counts were analysed using one-way ANOVA with multiple pairwise comparison corrected by Bonferroni. The majority of reported molecular analysis compares females aged 20 days and 80 days and males at aged 10 days and 40 days to reflect 20% and 80% of lifespan respectively. This is because males are not sexually mature until aged 8 days therefore at 10% of lifespan (5 days) are juveniles.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.exger.2019.03.008>.

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Author contributions

J.K.C conducted and designed the experiments as well as drafted the manuscript. J.S contributed towards the female comet assay data. L.M. designed the experiments and contributed to the writing of the manuscript.

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