



Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

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Journal of
Entomology
and
Zoology Studies

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2017; 5(4): 1877-1881
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Received: 02-05-2017
Accepted: 03-06-2017

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Relation between parental age and ROS levels in the parents themselves as well as the ensuing progeny in *Drosophila melanogaster*

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Abstract

The objective of the present study was to ascertain the ROS levels across two generations (parents and progeny) of *Drosophila melanogaster* populations simultaneously selected for faster pre-adult development and extended longevity, and their ancestral controls. The ROS levels were ascertained after the flies had experienced competition for food during the larval stage and were aged as adults for different lengths of time. The experiment was conducted in the month of January to March 2016.

The present study found significant difference in the ROS levels of the two populations but no effect of parental age was seen on the progeny in both the control and competitive condition with rest to ROS levels. In the parental generation, the female flies from the selected populations had an average 60 (relative Optical Density) of ROS compared to 128 (relative Optical Density) in their ancestral control populations. The study indicates that ROS production is dependent on the immediate environment rather than the long term genetic effects.

Keywords: Competition' ROS, aging, oxidative stress, CMH₂DCFDA, mortality, antioxidants, competitive ability, JB-control and FLJ-selected

1. Introduction

The phenotype of an organism is not only influenced by interaction of its genotype and the physical environment in which it grew up, but it is also seen to be dependent on the condition of parents [1]. In the development of progeny, parental effects are accepted to have an important role, but very less information is available about the significance of parental effects on adult life history traits, of which one important process is aging. *D. melanogaster* populations selected for late life reproduction were observed to produce population with increased longevity as compared to their controls [2-3]. Such kind of population could be called as gold mines to study demography, as well as suitable for studying physiological and genetic basis of aging. Aging, which is characterized by physiological decline, is a persistent inevitable process [3]. Due to high density of eggs in wild habitat, flies often face competition for food, growth and space. Competition can hinder the growth, and ultimately the reproductive success of an individual. Thus it serves as a selection pressure driving differential reproductive success and the evolution of traits that enable organisms to use resources such as habitat, nutrients and others variably compared to their competitors [4].

Numerous theories describe the significance of ROS (reactive oxygen species) we wish to discuss some of these here in order to understand the importance and relation of ROS with aging such as interaction of ROS with macromolecules and Hsps, and their effect on physiology of living systems with their overtime production [5].

The hypothesis on oxidative stress is considered to be one of the central explanations for aging process. Its central idea is that reactive oxygen derivatives and prominent free radicals released from the ETC (electron transport chain) of mitochondria, result in macromolecular oxidative damage leading to loss of their function and finally resulting in cellular, organ and finally organismal senescence or physiological decline [6].

Hydroxyl radicals, superoxides, hydrogen peroxides, are some of the important ROS (reactive oxygen species) that are produced as by-products of normal cellular metabolism. Accumulation of these leads to damage over time. Diminished activity of antioxidant defenses due to increased production of free radicals, also leads to decline in the activity of systems that

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repair oxidative damage. Numerous studies have supported oxidative stress theory, providing evidence that there is gradual build-up of oxidative damage to cellular molecules in *Drosophila* with advancing age [6-7, 8]. Various cellular defenses are present within an organism for quenching ROS, among them most necessary defense against reactive oxygen derivatives are provided by the group of enzyme superoxide dismutase (SOD) [5]. Super oxide dismutase usually exists in two forms as, Cu/ZnSOD present in the outer mitochondrial space and cytoplasm [9], and MnSOD which exclusively resides in the inner mitochondrial space inside the eukaryotic cells. An extracellular Cu/ZnSOD is also found in many species. SOD converts superoxides to H₂O₂ and O₂ generated in the mitochondria. Then catalase enzyme (abundantly present) converts H₂O₂ (hydrogen per oxide) to H₂O (water) and O₂ (oxygen molecule). But with the passage of time the ability of cells to clear ROS and repair oxidative damaged macromolecules declines, which results in accumulation of oxidative damaged products [6]. Mitochondria turn out to be a key destination of oxidative damage with aging, as it is the major producer of ROS in the cells [10]. *Drosophila melanogaster* (fruit fly), *S. cerevisiae* (yeast) and *Mus musculus* (mouse) are widely used in studies related to understand the role of superoxide dismutase enzymes and ROS production in aging and longevity regulation because of the powerful molecular and genetic tools available [5].

An increased level of metabolism leads to increased production of reactive derivatives and thus resulting in faster aging and shortened life span, this is in accordance to the free radical theory.

According to published literature, long-lived lines of flies have the equal metabolic rate ($\mu\text{l O}_2/\text{mg/h}$) as short-lived lines and thus increased lifetime metabolic potential ($\text{ml O}_2/\text{mg/lifetime}$) [17]. In the similar manner, no correlation has been found between the activity score at young age [18], or the average lifetime activity score [11-12] and lifespan. If there was a tight connection between energy expenditure and lifespan then negative correlations might be expected. But this idea of a tight connection has been abjured, even with the support of the free radical theory [13]. *D. melanogaster* possess Mn mitochondrial SOD, cytosolic Cu/Zn SOD and (GR) glutathione (GSH) reductase and CAT, but do not possess GSH peroxidase (GPX) [14]. SOD converts O₂⁻ to H₂O₂, while CAT and GPX convert H₂O₂ to H₂O. The conversion of the oxidized form of GSH to the reduced form is done by GR. By modulating (increasing or decreasing) the activity of these enzymes, in accordance with the free radical theory, should enhance or cutback longevity, respectively. These activities can be reduced either by creating mutant flies or by chemical inhibition *in vivo* to achieve a lowered enzyme activity. The activity can also be raised to some extent using transgenesis. No attempt has been made to reduce the SOD activity in *D. melanogaster* *in vivo* [12]. Only a small activity of SOD is sufficient to survive in normal conditions but these studies indicate that adult life processes are affected/hampered when SOD is absent [12]. It is interesting to note in this regard that flies have evolved maximum SOD-related genes and one of the most metabolically active tissues in the metazoans flight muscle [15].

It can thus be summarised that with increase in chronological age, bulk of oxidative damage and inactivation of enzymes occurs. Reactive oxygen derivatives damages major cellular macromolecules *viz.* nucleic acids, lipids, proteins, carbohydrates, and these damaged oxidative products accumulate [5, 15-16].

One important relationship between longevity and oxidative stress resistance has to be told as this is one of the important basis of this work. Interference that increased organismal life span is found to be associated with increase in stress resistance. Laboratory populations of *Drosophila* for increased longevity, correlates genetic selection with increased stress resistance and higher expression of stress related genes like hsp22 and SOD [17]. In organisms like *C. elegans*, *Drosophila* and *Mus musculus*, single gene mutations are found to increase longevity and resistance to oxidative stress [18].

In this paper, we made an attempt to gather evidence from *Drosophila melanogaster* research, a most sought after model in oxidative stress and aging experiments that what is happening to ROS levels in competitive conditioned flies (facing high density) as more or else they are capable of maintaining their system to cope up with the harsh conditions of the scarcity of food, better, and improved their survival strategy as it was found, (data not shown here) that they were capable of surviving more than the control conditioned flies (facing low density i.e. sufficient food). Compilations of present study results lead to an assumption, if there is a certainty that free radicals are the primary cause of aging, undoubtedly it possibly have detrimental effect on organisms and hence turning cornerstone in understanding of aging.

In this experiment, we were interested to know what would be the ROS levels in the flies facing stress of competition for less food and high larval density belonging to differentially aged parents as in our knowledge it has not been reported till date and observe the ROS production in the longer living flies (FLJ) and the control population (JB) in two generations.

2. Materials and Methods

The aim of present study was to have an insight into plausibility, whether with increase in age, ROS levels increased significantly or not at parental level (both control and selected population) and if there is any causal link of it in their progenies? Also level of ROS varies or not in progenies of both the condition i.e. raised in control (low density condition) and competition (high density condition) environment? For this we used 2 populations of *Drosophila melanogaster* selected for divergent traits, progenies were obtained from various chronologically aged parents separately to see the level of ROS. The experiment was conducted in the month of January to March 2016 in Evolutionary Biology Lab of Department of Zoology, University of Delhi, Delhi.

2.1 *Drosophila* Strains and Animal Husbandry/Rearing Conditions and Treatments

In this experiment 2 types of population were used the control designated as JB and the selected one designated as FLJ [19]. JBs are known as Joshi Baselines. All the adults that emerge by 12th day from the day of egg collection are transferred to clean plexiglass cages. The selected population FLJ (faster pre adult development late reproductive Joshi baselines) was derived from JB. Only the first 15-20 emerging flies were transferred from each vial breeding cages by maintaining a close watch on the emerging flies. The reproducing adults were allowed to age in the cages till the population is reduced to about 40-50% in one of the sister cages. Eggs for starting the next generation were collected from these flies (i.e. both ends selection pressure has been given for faster development and late reproduction). Both populations were maintained at SLC [19-20].

The fly stocks were kept on banana-yeast-jaggery based food.

In this experiment all fly stocks (different ages) were raised in vials with 6 ml standard media (SM) as mentioned in Mishra and Shakarad (2016)^[19] and Handa *et al.* (2014)^[20], food at standard laboratory conditions (SLC) of 24:0 light: dark cycle, 24 °C temperature and 75% RH humidity. The emerging adult flies were transferred to pre-labeled cages and were allowed to age according to the experimental protocol for different ages. In addition to the progenies sufficient flies were also collected on predetermined age of the parents (3, 10, 20, and 30 for both populations with additional 45 day for selected population) for their ROS quantification. Subsequent to this adult flies (progeny) subjected to competition (200 eggs /2ml media vials) and their parallel control condition flies (50 eggs/2ml media vials) were also pooled across variable ages of parents.

2.2 ROS Quantification Assay

ROS estimation was done using CM-H₂DCFDA protocol^[21] with minor modifications. At every age (of chronologically aged parents and their respective progeny of control and competitive condition), group of 10 flies were transferred to 1.5 mL micro-centrifuge tubes (MCT), was homogenized by using mini hand held homogeniser (BenchTop lab systems) in 500 µl of 0.1M PBS (PBS was prepared using HPLC-grade water) with 10µM CM-H₂DCFDA (Invitrogen) solution. 10

µM H₂DCFDA dye was prepared using DMSO immediately before dilution in PBS. Homogenised samples were centrifuged for 5 minutes at 14,000 rpm at 4°C. 100 µl of supernatant was extracted for assay, was then loaded to a black 96-well plate in sets of 3 replicates and the fluorescence intensity was measured using spectrofluorometer (Tecan) after 20 minutes of incubation at excitation of 485 nm and emission wavelength of 538 nm respectively and cut-off was 530 nm.

2.3 Statistical Analysis

Statistical analysis was done using the one-way ANOVA using the SPSS software (SPSS Inc. version 16.0).

3. Results

3.1 ROS Accumulate in Parental flies

The ROS production varied significantly for selection ($F_{1,2} = 794.928$, $p = 0.001$), age ($F_{4,8} = 117.602$, $p = 3.8572E-07$) and gender ($F_{1,2} = 79744.793$, $p = 1.254E-05$). The other interactions found to be significantly different were selection x gender ($F_{1,2} = 545.161$, $p = 0.001$), selection x age ($F_{3,6} = 18.810$, $p = 0.001$), gender x age ($F_{2,4} = 7.116$, $p = 0.009$), selection x gender x age ($F_{3,6} = 9.936$, $p = 0.009$).

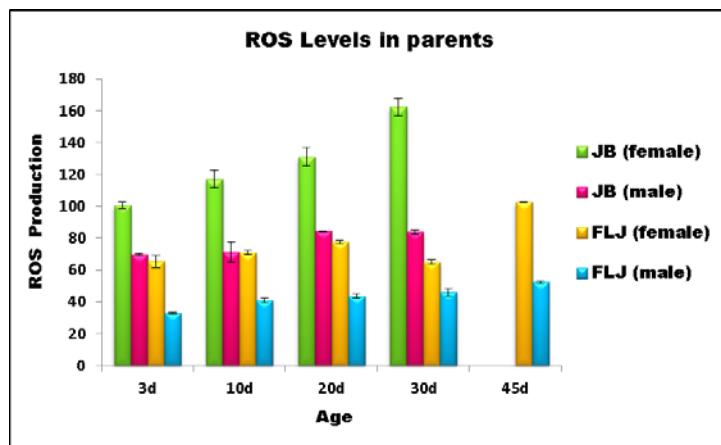


Fig 1: Mean (± s.e.) ROS levels in female and male parents of different ages from control and selected populations.

3.2 ROS Accumulate in Progenies in Control and Competitive Condition

The ROS production also varied significantly in progeny for selection ($F_{1,2} = 750.76$, $p = 0.023$), with age ($F_{4,10} = 13.566$, $p = 0.000$) and gender

($F_{1,2} = 600.142$, $p = 0.004$). Significant difference was observed with variable density ($F_{1,2} = 61.343$, $p = 0.004$). There was no specific pattern observed with increase in age of parents as ROS accumulates in progeny.

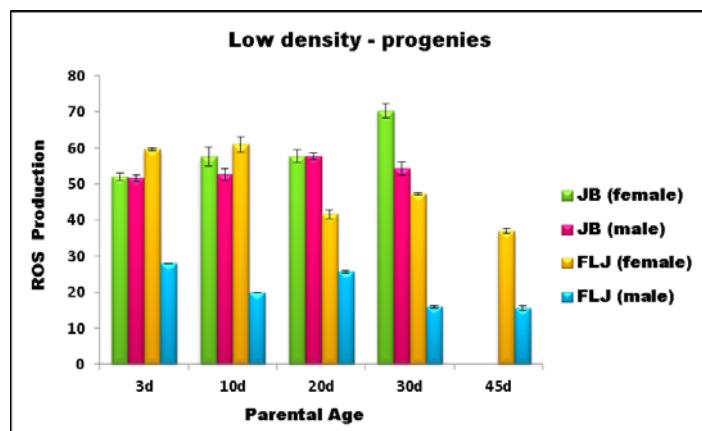


Fig 2: Mean (± s.e.) ROS levels in female and male progenies from differently aged parents in low density (control condition).

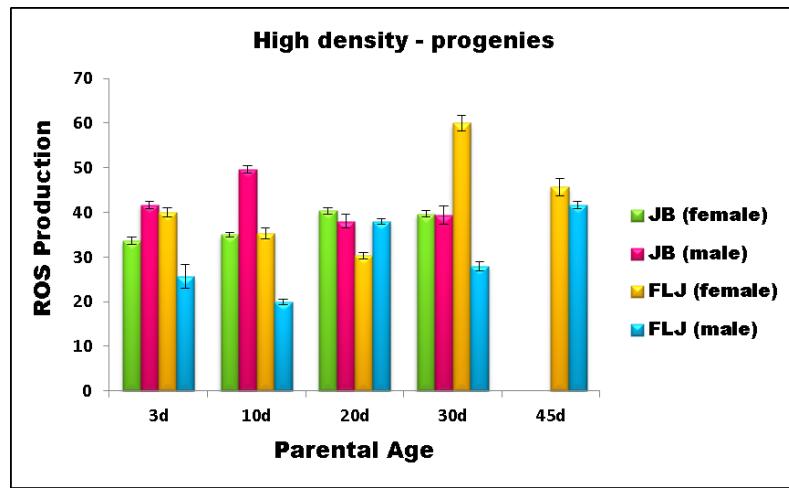


Fig 3: Mean (\pm s.e.) ROS levels in female and male progenies from differently aged parents in high density (competitive condition).

The other interactions found to be significantly different were selection x gender ($F_{1,2} = 210.830$, $p = 0.043$), selection x density ($F_{1,2} = 1814.76$, $p = 0.014$), selection x age ($F_{3,6} = 24.793$, $p = 0.012$), gender x density ($F_{1,2} = 83.856$, $p = 0.002$), selection x gender x density ($F_{1,2} = 1681$, $p = 0.015$), selection x gender x age ($F_{3,6} = 82.054$, $p = 0.002$), selection x density x age ($F_{3,6} = 39.337$, $p = 0.006$), gender x density x age ($F_{4,8} = 7.409$, $p = 0.004$) and selection x gender x density x age ($F_{4,8} = 35.796$, $p = 0.007$).

Seemingly, in the progeny flies (who were of similar ages) but belongs to differentially aged parents ROS accumulates followed no specific uniform pattern among them.

4. Discussion

The present study suggests that with increasing parental age ROS level increased, and with advancing age, accumulation of ROS increased which is a known important cause of aging in species ranging from lower organisms (*C. elegans*) to *Drosophila* and tolarger ones like (*Homo sapiens*) [22; 23-24]. The female flies were observed to have an increased ROS production perhaps due to increased oxidative stress. To quantify ROS in flies, CM-H₂DCFDA dye was used which is the cell permeate reporter and a common indicator of peroxide activity that is cleaved by cellular esterases and becomes fluorescent upon oxidation by cellular ROS [24]. Control conditioned flies showed increase in ROS levels, presumably reflecting poor up-regulation of antioxidant defenses. Male flies have lesser ROS levels but have statistically significant decrease in ROS levels than females. As oxidative stress results in damage to energy macromolecules (lipids, carbohydrates, proteins) and nucleic acids (DNA/RNA) its role is being implicated in many diseases, degenerative conditions and aging [25]. Hence this might be the key cause of increase in ROS level at parental level. But the ROS levels in progenies were observed to be non-significant which were approximately similar in progenies of different chronologically aged parents. Suggesting that age of the parents and their ROS level might have no relation with the damage occurred (here in terms of ROS levels) found in the progenies, it can be thus assumed that older parent's progeny will be more prone to the damage or will have hiked levels of their ROS accumulation. Also, non-uniform pattern of ROS was observed with the increasing age of the parents it can be suggested that it is purely a function of progenies life or vital activities such as fecundity and longevity and not linked with parental age/damage. Most

of the studies done by manipulating the level of antioxidant enzymes indicated that a certain percentage of its activity is required for longer lifespan, provided no powerful oxidative stress is encountered [12], in lieu of this, we restricted our experiment so that no additional stress was given to populations tested. In simple words, the normal enzyme activity level is sufficient to counteract with basal levels of free radical's production seen nearly for all the progenies belonging to different parents. It seems, therefore, antioxidant enzymes might have a minor role during normal aging process and are not a strong deciding factor of individual longevity even if they are widely used in stressful conditions [12]. Differences in response to selection were observed between control and selected flies so reduction in ROS level can be hypothesized as the basis for lifespan extension in response to selection [27]. If the organism is stressed it takes the advantage of antioxidant enzymes and supplements, the activity provided by overexpression which might be useful to combat stress [28]. The key finding of the present study indicate that oxidative damage increases with age (parental data) on an average and is lower in selected lines when compared to their parallel controls. There are two possible models at present to reconcile these results. First, there are different types of oxidative damage—the cell generates different types of ROS and thus contains several clearing and repair pathways. Oxidative damage might cause aging in all species, but the key types of oxidative damage may be only indirectly affected by manipulating SOD enzyme levels. This suggests that superoxide is not the key ROS causing aging, but one or more other species of ROS does directly limit life span. Second, we have done experiment on flies which belong to different phylum from nematodes and mammals, and oxidative damage accumulation correlates with age in all species [14] perhaps oxidative damage limits life span to a greater extent in flies than in the other species. Also it can be said that the accumulation was random in progeny because one has differences in performing physiological activities. From present results, we accept that key evidence establishes a direct causal link between oxidative stress and the rate of aging, as at parental level ROS production increased significantly but still there exists an enormous gap in understanding the etiology of aging for the progenies of aged parents. Further, the reason for variation in the rate of aging/senescence, between both (parental control and selected) populations and for genders, need validation with the aid of molecular experiments which will be useful to find

whether these results can be concluded for better understanding or not in future.

5. Conclusion

This study concludes that ROS levels are increased with the increase in the age of the organisms as it is the major cause of aging. When it comes to progeny, there is no relation between the ROS levels of the parents (damaged done in the parents) with the quality of their progeny, i.e. it is totally dependent on their activities and provisioning of resources. The two kinds of populations used in this study indicate that the ROS production is dependent on the immediate environment experienced rather than the long term genetic effects.

6. Acknowledgements

This work was supported by a grant from the Department of Zoology, University of Delhi. We are also thankful to other lab members and lab attendant for their assistance.

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