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Effects of Reactive Oxygen Species on Life History Traits of *Caenorhabditis elegans*

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Effects of Reactive Oxygen Species on Life History Traits
of *Caenorhabditis elegans*

by

Samson William Smith

A thesis submitted in partial fulfillment of the
requirements for the degree of

Master of Science
in
Biology

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Abstract

Evolutionary life history theory predicts that tradeoffs among fitness-related phenotypes will occur as a result of resource limitations and/or physiological constraints. Such tradeoffs are defined as the cost(s) incurred on one component of fitness (e.g., reproduction) by the increased expression of another fitness-related trait (e.g., longevity). Only recently have researchers begun to investigate the mechanistic bases of life history tradeoffs. A recent proposal is that reactive oxygen species (ROS) have a central role in shaping life history traits and tradeoffs. Research on disparate animal taxa has highlighted strong correlations between oxidative stress resistance and fitness-related life history traits, for example. Here, I use the model organism *Caenorhabditis elegans* to test several hypotheses concerning the effects of ROS on life history traits and the manifestation of life history tradeoffs. Additionally, I use heat stress and an alternate food source to explore the responses of life history traits to other forms of physiological stress. Relative fitness and other traits related to reproduction were found to be affected in mostly negative ways by increasing oxidative insult. Lifespan was surprisingly unaffected by oxidative stress, but was modified by temperature. *In vivo* ROS levels as measured by fluorescent microscopy reveal a tradeoff between antioxidant production and reproduction in this species.

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“Life ain’t nothin’ but a funny, funny riddle.”

– John Martin Sommers

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Chapter 1: Introduction

An organism that is capable of unlimited reproduction over the course of an infinitely long lifespan exists neither in nature nor in the laboratory. Although this might seem a trivial observation, the absence of such a “Darwinian demon” (c.f., Reznick et al., 2000) suggests that there is a constraint on fitness-related traits preventing their boundless evolution. Constraints on fitness come from both extrinsic and intrinsic forces (2). Extrinsic selective pressures on life history traits originate from environmental sources including predation and food acquisition. For example, strong predation may result in faster maturation time and smaller size at reproduction (3). By contrast, intrinsic sources of selection on life history traits are often associated with the concept of life history tradeoffs (4). Life history tradeoffs exist when increases in one component of fitness result directly or indirectly in the decline of another component of fitness. The negative correlations between such fitness components will prevent the successful maximization of both traits simultaneously; the effect of these negative genetic correlations is that both affected traits are constrained to a local maximum within an phenotypic adaptive landscape (5,6).

The proximal mechanisms or constraints that determine the existence and magnitude of life history tradeoffs (i.e., intrinsic sources) are not well understood. Pearl (7) proposed that some finite, or ‘vital’, resource was being depleted over the lifespan of an organism that, when fully expended, led to death. This hypothesis makes intuitive sense with respect to specific metabolic rate and maximum lifespan as there has been shown to be a strong negative correlation between these two traits in a wide range of

mammalian taxa (8). The idea that some aspect of metabolism negatively impacts life history traits, primarily senescence, is now incorporated into the oxidative stress theory of aging. This theory expands upon the mitochondrial biological clock/free radical theory of aging put forth by Harman (9) and states that the mere act of energy production causes senescent decline of organisms through the inevitable production of reactive oxygen species (ROS) (10). ROS are known to cause DNA lesions, lipid peroxidation and other forms of damage that are expected to reduce fitness in age-related manner (11). The production of ROS may thus present a cost in higher energy production (directed toward antioxidant defense) and has recently been hypothesized to play a key role in life history tradeoffs (12). In support of this idea, metabolic rate is also known to correlate well with antioxidant protein expression (13). Life history tradeoffs are well discussed in theoretical literature although these tradeoffs are not always revealed in laboratory experiments (1). Stearns (1989) identified two mechanisms that would generate non-negative genetic correlations, both of which rely on internal environmental conditions such as genotype specific growth rate and resource allocation. In short: negative correlations might only be revealed in poor or stressful conditions.

Caenorhabditis elegans is a small free-living soil nematode that was first isolated in decaying plant material in Bristol, UK, and has since been repeatedly isolated mainly from compost sites near human populations. This small (about 1.5mm in length) transparent and primarily hermaphroditic nematode quickly became a widely used model organism after one enterprising researcher, Sydney Brenner, suggested that it was ideal for studies linking genetics to neurology and behavior (14). Since then, *C. elegans* has

served as a model for studies ranging from developmental genetics to environmental biomonitoring (15) and aging (16), and has been fully developed as a genetic model organism. Male homosexuality has also been documented in a wild isolate making it perhaps one of the only small invertebrates to have been observed engaging in this behavior (17) and illustrates the complex behavioral and genetic similarities it may share with more physiologically and genetically complex animals. The short life cycle of *C. elegans* also contributes to its value as a general research model and makes this species an ideal model for investigating outstanding questions in evolutionary life history theory.

Here, I examined the role that ROS might play in mediating life history tradeoffs in *C. elegans* by subjecting individual hermaphrodites to increasing levels of chronic oxidative stress caused by paraquat exposure (Chapter 2). I also performed additional assays using a combination of oxidative stress and mild heat stress in a further attempt to reveal negative correlations among life history traits. I found few significant negative fitness-related trait correlations at either temperature treatment as oxidative stress increased and thus found little support for the hypothesis that exogenous ROS mediates life history tradeoffs. I further interpret these findings within the context of data on *in vivo* ROS levels. Both oxidative and heat stress did produce substantial effects on certain individual life history traits. For example, the most surprising finding of this study was that although temperature had a significant effect on average nematode lifespan, the level of oxidative stress had no effect on this trait. Furthermore, I showed that worms fed UV-killed bacteria had severely reduced fitness as oxidative stress increased compared to worms fed living *E. coli*. These results suggest that *C. elegans* may receive significant

antioxidant supplementation from living bacteria, or that living bacteria initiate an innate antioxidant response in these worms, either of which results in increased fitness when experiencing chronic oxidative stress. Finally, I summarize the major findings of this thesis and discuss its implications as well as work in progress and directions for future research (Chapter 3).

Chapter 2: Effects of Reactive Oxygen Species on Life History Traits of *Caenorhabditis elegans*

Introduction

Evolutionary life history theory predicts that tradeoffs among fitness-related phenotypes will occur as a result of resource limitations and/or physiological constraints (4,12,18,19). Life history tradeoffs are typically defined as the cost(s) incurred on one component of fitness either concurrently or subsequently by the increased expression of another trait in a kind of zero-sum game. Without such tradeoffs we should expect the evolution of a “Darwinian demon” (c.f, Reznick et al., 2000), or an organism that could reproduce throughout its long lifespan and avoid age-correlated senescent decline. Commonly demonstrated tradeoffs include the so-called ‘costs of reproduction’ whereby lifetime fecundity is negatively correlated to other traits such as somatic maintenance (18). In this example, individuals who produce more offspring should have predictably shorter lifespans, accelerated aging, and/or be more susceptible to stress than individuals who have fewer offspring (20,21). In contrast, researchers have also reported positive or variable correlations between traits where classic theory would predict the existence of strong negative correlations (1,22). There has been much debate as to why the negative correlations predicted by theory are not clearly obtained in empirical studies (e.g., 24). With specific regard to laboratory studies of tradeoffs, positive correlations among life history traits may be obtained when resource budgets are expanded as is the case under ideal laboratory conditions with unlimited resources and optimal environmental conditions; however, reducing these resource budgets might illuminate true tradeoffs (1,24). Accordingly, negative correlations among life history traits have been revealed

experimentally by manipulating resource intake, brood size, or modulation of other stressors while tracking correlations among life history traits (18,21,25,26).

The currency or constraining resource that mediates life history tradeoffs has been hypothesized to be energetic, hormonal, and/or the result of more direct negative effects on one trait from the expression of another trait (see reference 4 for a detailed review on proposed physiological causes of life history tradeoffs). A growing body of literature suggests that reactive oxygen species (ROS) may play a central role in many aspects of life history evolution (27), including tradeoffs and aging. Indeed, ROS have been proposed to directly mediate life history tradeoffs by initiating oxidative stress, initiating macromolecular damage that would presumably require the allocation of resources away from reproduction and to somatic maintenance (12,27). Oxidative stress may be defined as the oxidative damage to macromolecular cellular components resulting from an inequity between the production of ROS and the production and/or procurement of protective antioxidants (1). The role of general oxidative stress in age-correlated cellular damage was initially highlighted by Harman (1956) whose hypothesis was an extension of Pearl's (1928) rate of living theory. Harman's proposal implicated mitochondria, the primary source of endogenous ROS, as a major cause of senescence through the production of free radicals by the electron transport chain (ETC). This hypothesis was later incorporated into a more general oxidative stress theory of aging (11,29). ROS are a natural byproduct of aerobic energy (ATP) production that occurs via the mitochondrial ETC (30). Endogenous ROS production from mitochondrial respiration has been empirically shown to result in the buildup of age-correlated nucleic acid, lipid,

carbohydrate and protein damage (31–36). For example (32) demonstrated high steady state levels of oxidative damage to both mitochondrial and nuclear DNA as indicated by the detection of the oxidatively modified guanine base-derived 8-oxo-dG in rat liver. In a model where ROS mediate life history tradeoffs, an organism must satisfy the requirements of its dependence on ATP production to fuel cellular functions and also be capable of regulating the levels of detrimental ROS byproducts thereby creating a possible tradeoff between these somatic maintenance (systems inhibiting the age-correlated decline of somatic tissues) , growth and reproduction.

Research on ETC dysfunctional mutants has shed light on the link between mitochondrial function (e.g., ROS generation) and life histories (37–42). For example, mutations affecting protein subunits of the ETC have been shown to have significant, mostly detrimental, effects on various components of fitness, including development, lifespan, fecundity and responses to stressors such as heat, oxidative stress, and UV irradiation (43). These effects are thought to result from increased mitochondrial ROS production. In studies of the nematode, *Caenorhabditis elegans*, short-lived ETC mutants have shown increased sensitivity to redox cycling compounds. Conversely, a trend between increased resistance to pro-oxidants and increased longevity has been established (42,43); for example a long-lived *C. elegans* ETC mutant, *isp-1*, is resistant to paraquat, a ROS-generating herbicide (44). Many other studies have highlighted the link between fitness-related traits and either ROS production or oxidative susceptibility and provide support for the idea that oxidative stress plays a central role in lifespan determination and that it may be at the heart of life history tradeoffs (11,12,27,41).

However, ROS and other free radicals are also known to have beneficial and vital intra- and inter-cellular roles in the immune system and as second messengers in signal transduction cascades (36,45,46). Studies that have modified antioxidant levels have produced counterintuitive results with respect to lifespan (47–52), but generally support the idea that antioxidants (both exogenous and endogenous) protect against high and acute oxidative stress (49,53). Taken together, these findings suggest a complex relationship between balancing fitness by minimizing oxidative damage and maintaining the beneficial roles associated with ROS signaling.

Here, I investigated the link between oxidative stress and life history tradeoffs by measuring life history characters in *Caenorhabditis elegans* nematodes exposed to two stressors, oxidative stress and thermal stress. For the oxidative stress treatments, I exposed nematodes to paraquat, alternatively known as methyl viologen – an herbicide that generates superoxide anions *in vivo* (54,55) and is commonly used experimentally to test oxidative stress resistance (56–58). Next, I examined whether and how life history traits would respond to a combination of oxidative and thermal stress. Increased temperatures (relative to the lab-determined optimum of 20 °C) are known to affect development, fecundity, and lifespan in *C. elegans* (22). I subjected worms to 25 °C, a temperature at which wild-type nematodes are known to have accelerated development resulting in a shorter period of egg-laying and a significantly shorter lifespan (22). Unfortunately, the results of these experiments are confounded by differences in nematode diet, an issue that is being addressed as part of ongoing research (see Chapter 3).

Additionally, I conducted *in vivo* measures of ROS levels for individual nematodes exposed to each oxidative stress and temperature regime to understand the effect of these stressors on cellular ROS levels. These experiments were conducted using nematodes experiencing identical dietary regimes. This portion of my study was motivated by the work of Tawe et al. (1998) and (59) showing the effect of age on physiological responses to stress. Tawe et al., 1998, subjected *C. elegans* to short periods of high concentrations of paraquat and found an increased expression of antioxidant proteins in larval stage worms relative to untreated control worms. Interestingly, they found no difference in antioxidant levels in mixed-age cohorts that were exposed to the same level of oxidative insult; i.e., older worms exhibited a dampened transcriptional response to acute oxidative stress compared to larval worms. Based on these findings, I hypothesized that net *in vivo* ROS levels should decline with increasing paraquat treatment (as a result of the upregulation of antioxidant mechanisms), at least until treatment levels beyond which nematodes could no longer balance both reproduction and somatic maintenance. Additionally, I expected that ROS levels within each stress treatment would be lower in larval worms compared to adult worms due to the increased antioxidant production of the former. In summary, these experiments were expected to reveal life history tradeoffs by reducing the animals' total resource budget and also to indicate how animals prioritize their resources under increasing levels of physiological stress.

In stark contrast to my expectations, I found that increasing levels of oxidative or thermal stress failed to reveal the life history tradeoffs predicted by theory. In fact,

correlations between phenotypic traits (e.g., lifespan vs. late reproduction) became strongly positive under the most stressful treatment for the fitness-related traits that I measured. Both forms of stress did, however, negatively impact individual reproductive fitness traits as stress levels increased. On the other hand, average nematode lifespan was surprisingly resilient to the forms of stress applied in this study. In agreement with my predictions, *in vivo* ROS levels were indeed lower in larval worms relative to adults from the same treatment cohort and in larval worms experiencing the highest oxidative stress treatment. This result indicates that strong antioxidant systems, which may have been supplemented through dietary means, are capable of staving off the accumulated macromolecular damage associated with senescence and that chronic oxidative stress impacts reproduction more than lifespan in this system as illustrated by resultant life history assay data. This final observation suggests that a life history tradeoff mediated by ROS occurs between reproductive fitness and antioxidant production but not with lifespan, as was expected.

Materials and Methods

Nematode Strains and Culture Conditions

The Bristol N2 strain of *Caenorhabditis elegans* was used for all experiments. Nematodes were grown on 60 mm Petri plates containing NGM media with streptomycin at 20 $\mu\text{g}/\text{mL}$ and seeded with OP50-1 *Escherichia coli* as a food source. Nematode and bacterial stocks were obtained from the Caenorhabditis Genetics Center (University of Minnesota, St. Paul, MN). Prior to experimentation, additional nematode stocks were stored at -80C following standard protocols (61) and thawed as needed for all assays. Worms were age-synchronized by standard bleach treatment prior to each assay and

grown at either 20 °C or 25 °C, as specified. Samples were acclimated to their respective temperature and food regimes for 2-3 generations prior to each experiment in an effort to minimize possible maternal effects.

Most of these assays were conducted using live *E. coli* as a nematode food source, which is standard practice in laboratory studies of *C. elegans* (Steirnagle 2011). I performed one life history assay at 25 °C using live *E. coli* as a food source and one at 20 °C using UV-killed bacteria (see below for further detail). For any assay, NGM plates were seeded with 100 µL OP50-1 *E. coli* that had been cultured at 37 °C overnight in LB broth. Plates in the second life history assay (20 °C) were incubated at 37 °C for 4 hours to grow a light bacterial lawn and were then UV sterilized (Logic Class II, Type A2 biosafety cabinet, Labconco, Kansas City, MO) for 2 hours at room temperature. Plates were stored overnight at room temperature and any plates with obvious signs of contamination, cracks or still-living OP50-1 colonies were discarded. Living colonies of OP50-1 can be easily identified by colony morphology after being allowed to sit overnight at room temperature.

Paraquat Treatments

Paraquat (PQ), also called methyl viologen dichloride, is an herbicide commonly used to experimentally generate exogenous oxidative stress by generating superoxide anions *in vivo* (10,56–58). For all assays in this study, NGM plates were supplemented with one of four PQ treatment levels: 0, 0.1, 1.0 and 2.0 mM PQ, hereafter referred to as control, low, medium and high treatment levels, respectively. These levels refer to the concentration of the PQ solutions rather than the final concentration in the media. Two

hours prior to transferring worms, 0.5 mL of the appropriate PQ solution was added to each plate containing 15 mL solidified NGM and pre-seeded with OP50-1. PQ solutions were diluted in sterile water, which was also used as the control treatment. PQ solutions were stored at 4 °C covered in foil and made fresh prior to each assay. Nematodes were first exposed to their respective treatments on the unseeded plates following age-synchronization (as eggs). PQ treatment concentrations were decided upon in preliminary life history assays conducted using live *E. coli* which revealed that PQ levels of at least 5 mM (stock) were required to induce a high proportion of larval arrest (data not shown). PQ levels that dramatically reduced or prevented the timely progression of worms through larval stages to reproductive maturity were not considered germane to this study.

Temperature Treatments

I conducted all preliminary assays and the first life history assay at 25 °C ± 2C. This temperature is commonly used in the *C. elegans* literature (14,47,53,62–65) as it allows for a faster generation time and reduced lifespan relative to 20 °C, which is another commonly used thermal regime in *C. elegans* studies. Twenty °C is considered to be the thermal optimum for *C. elegans* based on thermal preference experiments measuring intrinsic rate of growth (e.g., (66), although this species has broad thermal tolerance and exhibits remarkably weak preference for any particular temperature when allowed to explore a thermal gradient (67). I therefore repeated the life history and other assays at 20 °C. The temperature at which each experiment was conducted is denoted in the results.

E. coli Food Source

Food source type and temperature are noted for each assay as each is discussed.

Life History Assays

To determine the effects of the above treatments on fitness-related traits, two life history assays were performed following the general method of Estes et al. (2004). I conducted one life history assay using live *E. coli* at 25 °C and a second trial at 20 °C using UV-killed *E. coli* (unless otherwise noted). Single L1 stage larvae were transferred to individual Petri plates. These focal nematodes were then transferred daily to fresh plates for at least five days following the first reproductive event to separate them from their progeny. However, for logistical reasons, worms were allowed to stay on plates for the remainder of their lives if no progeny were detected for the previous 24 hour period. Average total fecundity and reproductive schedules were obtained by counting surviving progeny on Toluidine-dye stained plates. Only worms that had successfully hatched from their eggs were counted. “Early fecundity” was calculated as the number of surviving offspring produced during the first and second days of plate transfer (which coincided with the first two days of reproduction in control worms), and “late fecundity” was the sum of the remaining days of reproduction. These traits capture the fitness effect of any change in the reproductive schedule caused by the stress treatments. Conversely, “relative early fecundity” and “relative late fecundity” describe only changes in fecundity and ignore changes in the reproductive schedule. Relative early fecundity was calculated as the number of surviving offspring produced during the first two days of the reproductive period for each treatment, and relative late fecundity was calculated as the total number

of offspring for any remaining days of reproduction. For example, if a worm produced 0, 50, 50, and 0 offspring on the first, second, third and fourth days of transfer, respectively, its score for early fecundity would be 50. Its score for late fecundity would also be 50. However, the relative early and late fecundity for this worm would be 100 and 0, respectively.

Total lifespan was calculated as number of days from L1 arrest until death. Worms were checked daily for survival and scored as dead if unresponsive to prodding with a platinum wire. Data were censored for nematodes that had desiccated after crawling onto the side of a plate.

Data from the life history assays described above were used to calculate relative fitness (ω). Relative fitness was calculated following the basic approach of Keightley et al. (2000, eq. 2) and further described by Estes et al. (2011). Specifically, I computed relative fitness for each individual as: $\omega = \sum e^{-rx} l(x) m(x)$, where $l(x)$ is the number of worms surviving to day x and $m(x)$ is the fecundity at day x , and where r is the mean intrinsic population growth rate of the control treatment worms from the appropriate assay. The latter was calculated by solving Euler's equation, $\omega e^{-rx} l(x) m(x) = 1$, for r .

Finally, I quantified average correlations among the measured life history traits across PQ treatments to investigate whether the magnitude or pattern of these trait associations changed with increasing oxidative stress.

Maturation Rate

In preparation for measuring mitochondrial oxidant levels as described below, one additional set of measurements was performed as part of the 20 °C and 25 °C experiments and conducted using live *E. coli*. Time to first reproduction was assessed for control and PQ treated worms within each temperature regime by placing age synchronous L1 worms on individual plates and noting the hour at which the first egg(s) were produced. Beginning 30 hours after L1 arrest, nematodes experiencing 25 °C were surveyed hourly for eggs; hourly egg checks for nematodes experiencing 20 °C began 50 hours after L1 arrest. The start time for these hourly time points were determined from preliminary experiments (data not shown). Each treatment cohort was removed for scoring from the incubator for identical lengths of time to control for differences in incubation and ambient temperature. Eggs were identified visually under a dissecting scope. Once the first eggs were identified, the parental hermaphrodite was sacrificed and the plate was allowed to incubate at room temperature to confirm egg viability the following day.

Relative Mitochondrial Oxidant Levels

For the 20 °C and 25 °C assays conducted using live *E. coli*, mitochondrial ROS levels were assessed using MitoSOX Red (Invitrogen, M36008, Carlsbad, CA) by following the basic methods developed by Dingley et al. (2010) and modified by Hicks, et al. (2012). Briefly, the terminal pharyngeal bulb was imaged in MitoSOX Red-labeled young adult nematodes from each treatment group. MitoSOX Red was recently shown to quantify total levels of mitochondrial oxidants, rather than superoxide specifically, when

used with confocal microscopy (74). The terminal pharyngeal bulb was chosen for fluorescence imaging because it is an easily identified physical marker with a fluorescence level unaffected by the high dye uptake of lipid rich gut granules, and because the pharyngeal tissue is mitochondria-dense, remaining energetically active until senescence. Age synchronous worms were incubated for 24 hours in 10 μ M MitoSOX Red before being transferred as young adults to fresh NGM plates seeded with nonlabeled OP50-1 *E. coli*. They were allowed to feed for 1 hour, and paralyzed using a drop of 5 M levamisole prior to imaging and 5 μ L polystyrene beads on agar pads (adapted from original methods developed by Christopher Fang-Yen, pers. comm.). 10 μ M MitoSOX Red working solutions were prepared as part of the respective PQ treatments, which were made from a freshly prepared 5 M MitoSOX Red stock solution in DMSO. The PQ + MitoSOX Red solution was added to plates as described above (Paraquat Treatments). Images of the pharynx were acquired using a high resolution wide field Core DV system (Applied Precision™), equipped with an Olympus IX71 inverted microscope mounted with a Nikon Coolsnap ES2 HQ camera (Advanced Light Microscopy Core Facility, Oregon Health and Science University, Portland, OR). Images were captured at 60X magnification with a 0.15 second exposure time and 5 μ m Z-stack widths. Images were deconvolved prior to analysis. Terminal pharyngeal bulbs were manually encircled within each image and maximum pixel intensity was measured for this region within each z-stack slice using ImageJ software (NIH). This procedure was applied to 40 exposed and 10 unexposed (control) animals for each PQ treatment. The final pharyngeal bulb intensity values were calculated as the difference between intensity values for exposed and control worms. Replicate maximum pixel value was calculated as

the mean of all maximum pixel intensities. Finally, great care was taken to minimize technical error. For example, I exposed only one sample at a time to the microscope light source and used the shortest exposure time possible to avoid introducing variation due to breakdown of the dye.

Dyeing regimes in the 25 °C experiment were applied at either the time of feeding after L1 arrest (24 hour trial), or 24 hours past feeding after L1 arrest (48 hr trial). Assay timing was adjusted in 20 °C worms fed live *E. coli* as informed by the time to first reproduction results. Young worms were imaged 34 hours after L1 arrest and older worms were imaged 68 hours post L1 stage arrest. 25 °C ROS data were log transformed to correct non-normal distributions.

Statistical Analyses

All statistical analysis was performed in JMP version 9.0.2 (SAS Statistical Inc., Cary, NC). In cases where data were non-normally distributed, attempts were made to normalize data through transformation. When transformations failed to normalize data for particular traits, these data were analyzed both by non-parametric analysis (Wilcoxon/Kruskal –Wallis tests using rank sums) and one-way analyses of variance (ANOVA) tests. When the results of parametric and non-parametric tests did not differ, parametric statistic and p values are reported. These tests were performed to determine whether PQ treatment or temperature had a significant effect on individual life history traits. To determine whether traits differed among particular pairs of PQ treatment levels within each temperature regime, either the parametric Tukey-Kramer HSD test or the non-parametric Wilcoxon method was performed. Again, results of the parametric tests

are reported when both types of tests gave the same result. Finally, I characterized associations among life history characters measured on the same individuals by calculating Spearman rank correlation coefficients between each pair of traits following Estes et al. (2011).

Results

Toward the major goal of assessing the effect of exogenous oxidative stress on life history in *C. elegans*, I assayed life history phenotypes and *in vivo* levels of ROS in worms treated with increasing concentrations of PQ and maintained in one of two experimental conditions: 25 °C with a live *E. coli* food source and 20 °C with UV-killed *E. coli*. A subset of these assays was also conducted at 20 °C with live *E. coli*. Results are reported for each measured phenotype assayed under these experimental conditions, which I denote as “25 °C” and “20 °C” below. It has been shown previously that unknown metabolite(s) of OP50 *E. coli* result in an innate immune response through the insulin-like IGF-1 signaling pathway which results in an almost 2-fold upregulation of *sod-3* mRNA transcripts compared to nematodes grown in a monoxenic plated cultures of UV-killed OP50 (68). I reasoned that PQ’s prooxidant nature probably results in the death or cessation of growth of *E. coli* which serves as a nematode food source, which would lead to a concomitant reduction in the aforementioned metabolite(s). In agreement with these expectations, I observed that bacterial lawns proliferated outside the original seeding location in the control and low PQ level treatment regime but showed minimal or no such proliferation in the medium and high PQ treated plates. I therefore chose to test whether similar results for life history traits would be observed if worms were fed only

UV-killed OP50-1 by conducting a separate life history experiment. In this way, any effect that PQ treatments might have on the *E. coli* food source and any downstream effects on nematode life histories could be eliminated.

Total Reproductive Output

I counted individual daily progeny production for each worm in each PQ treatment to identify the effects of oxidative stress on reproductive output (fecundity) and the timing of the reproductive cycle.

25 °C

Worms treated with low and medium PQ concentrations experienced slightly increased reproductive output relative to control (no PQ) worms (Fig. 1A, Table 1). Animals in the low PQ treatment had more offspring (205.7 ± 6.0) than both the control (176.5 ± 4.7) and high PQ (178.7 ± 7.0) treatment level, but did not differ significantly from worms in the medium PQ treatment (198.9 ± 7.3) with respect to this trait (Wilcoxon $Z = -0.754$ $p = 0.45$). Reproductive output in high PQ treatment animals was not statistically different from that in control animals (see Wilcoxon grouping Fig. 1A). Variance in total reproductive output among treatments did not differ significantly (Levene's test, $F = 1.41$, $p = 0.24$).

20 °C

In contrast to results from the 25 °C conditions, total reproductive output in this assay showed a simpler, dose-dependent decline as PQ levels increased (Fig. 1B, Table 2). Low PQ treatment worms had slightly lower fecundity than control worms but did

not differ significantly from the control treatment (Tukey-Kramer HSD, $\alpha = 0.05$). While there was a statistically significant decline in total reproductive output observed in the medium PQ treatment and again in the high PQ treated worms (Fig. 1B, Table 2), I found no difference in variance among all treatments for total reproductive output (Levene's test, $F = 0.71$, $p = 0.55$).

Reproductive Schedule

To evaluate the effects of oxidative stress on the schedule of reproduction, I measured daily progeny production for each nematode in each PQ treatment maintained in the aforementioned assay conditions.

25 °C

Under normal lab conditions at 25 °C, the majority of *C. elegans*' reproductive output was achieved within a 48 hour period; however, an extended period of offspring production occurred over several additional days. Control treatment reproduction peaked on day 2 of reproductive period and declined to essentially zero by day 4 (Fig. 2A). Compared to the control, worms in the low and medium PQ treatments had near identical temporal patterns of reproduction while worms in the high PQ treatment experienced a delayed reproductive schedule (Fig. 2A). More specifically, worms in the low and medium PQ treatments had significantly more offspring on days 2 and 3 of reproduction while worms in the high PQ treatment had significantly fewer offspring on those days compared to the control (Tukey-Kramer HSD, $\alpha = 0.05$). Peak reproduction occurred on day 2 for all worms in the assay, with the low PQ treatment worms having the most offspring (140.6 ± 3.6) followed by the medium treatment (138.0 ± 5.4), control

treatment (120.2 ± 3.5), and worms in the high PQ treatment (87.7 ± 4.7). The third day saw a major (~60%) decline in reproduction for all treatment levels except the high PQ treatment, which maintained a similar reproductive output to day 2. By the fourth day of reproduction, reproduction in the high PQ treated worms was still slightly elevated, but reproduction was negligible in all treatments and had completely ceased by day 5 (Fig. 2A).

20 °C

Similar to previous findings (22), control worms at 20 °C followed a reproductive schedule that persisted for about 6 days with peak daily fecundity on day 3 (60.4 ± 2.8) (Fig. 2B). Low PQ treated worms had a similar schedule, yet a slight delay can be seen as low PQ treated worms had peak reproduction on day 4 (61.3 ± 2.6) rather than on day 3. Medium PQ treated worm reproduction also peaked on the fourth day of reproduction, although the peak was much lower than that of the other treatments (41.2 ± 2.7). The high PQ treated worms had low overall reproduction in addition to a markedly delayed reproductive schedule with all but one worm failing to produce offspring until the third day of reproduction in control worms. These high-PQ treated worms produced their peak number of offspring on the fifth day followed by a steady decline in offspring numbers. Notable is the large extension of the reproductive period for some high PQ treatment worms, with ~25% of the surviving worms continuing to produce surviving offspring until the thirteenth day and the last offspring being produced on the 16th day of the assay (after reproduction began for the control worms) (Fig. 2B). This pattern also diverges significantly from the 25 °C assay reported above (Fig. 2A).

Maturation Rate

To further characterize the effect of oxidative stress and temperature on the temporal pattern of reproduction, I measured the time at which worms produced their first surviving egg. This measurement will more precisely differentiate treatments in terms of their reproductive timing than the standard reproduction assays reported above. Further, I wished to know whether the 25 °C temperature treatment might induce shock response pathways that confer ROS resistance, which could culminate in a PQ level-by-temperature interaction effect on reproductive timing. Note that both assays were conducted using live *E. coli* as a food source.

25 °C

I found that increasing PQ treatment level was associated with increasingly longer times to first reproduction (Fig. 3, leftmost data points). Although control and low-PQ treated worms both produced their first surviving eggs at approximately 42 hours after L1 arrest, nematodes in the medium PQ treatment were significantly delayed by 4.12 hours (± 0.61) and worms in the high PQ treatment were delayed by 8.65 hours (± 0.64) compared to the control. Additionally, while reproduction in the low-PQ treatment worms was not statistically different than that of the control, it was slightly delayed relative to control. This result is somewhat unexpected and indicates that the increase in total reproduction was not due to faster development to reproductive age.

20 °C

Although the average time to first reproduction was substantially longer at 20 °C as compared to 25 °C (63.4 ± 4.0 vs. 42.4 ± 2.8 in control worms), the pattern of

differences among PQ treatments was almost identical at both temperatures (Fig. 3). At 20 °C, medium-PQ treatment animals had a mean 4.3 hour (± 0.48) delay in the amount of time to first egg lay, while high-PQ treatment worms had a 10.55 hour (± 0.52) delay relative to controls.

A linear regression model testing the effects of temperature, PQ concentration, and the interaction of those two factors revealed that there were significant effects of both temperature and PQ concentration on mean time to egg laying, but no significant interaction effects between the two factors (Table 3). Visualizing the reaction norms in Fig. 3 highlight the essentially linear shift in mean time to first egg laid for all worms measured at each temperature. In other words no environment-by-environment interaction was observed for this trait.

Lifespan

I calculated mean lifespan for all cohorts in each treatment condition to determine how temperature and/or level of oxidative stress might modify this trait. I expected to find a general decrease in lifespan at the higher temperature (25 °C) as shown previously (22). I also hypothesized that mean lifespan would decline within temperature treatments as oxidative stress caused by paraquat increased in agreement with the oxidative stress theory of aging (11).25 °C

There was no difference in mean lifespan among worms experiencing different PQ treatment levels (ANOVA $F = 0.49$, $p = 0.69$; Fig. 4A).

20 °C

The resilience of mean lifespan to increasing oxidative stress was again evident at the lower temperature and on UV-killed OP50 *E. coli* food source. Mean lifespan did not differ among PQ treatment level (ANOVA $F = 0.69$, $p = 0.56$; Fig. 4B). Similarly, variance in lifespan also did not differ among treatments (Levene's test, $F = 0.44$, $p = 0.72$). A qualitative comparison of survival curves for all worms at each temperature is shown seen in Fig. 5.

Worms that died from bagging (premature hatching of eggs in the gonad resulting in death of the parent) were not removed from lifespan analysis. The relative few instances this occurred in each treatment could not be analyzed for variation among treatments or temperature due to small sample sizes.

Relative Fitness

Using the life history data reported above, I calculated relative fitness for each PQ treatment cohort for each assay (Fig. 6). Because ROS lead to many types of macromolecular damage, I expected that relative fitness should decline with increasing oxidative stress in both experimental conditions.

25 °C

Worms subjected to the low PQ treatment level had the highest relative fitness (Fig. 6, Wilcoxon rank sum, $\alpha = 0.05$) There was no statistical difference in pairwise mean comparisons between control and medium treatments. The cohort exposed to the highest level of PQ had significantly lower mean relative fitness compared to any of the

lower PQ treatment levels even though mean fecundity was not statistically lower than the control group. Additionally, the variance in fitness of the medium-PQ treated worms was greater than that of the other groups (Levene's test , $F = 3.91$, $p = 0.0095$).

20 °C

Similar to the trend observed for total mean fecundity, worms incubated at 20 °C and fed UV-killed *E. coli* had declining relative fitness as PQ levels increased (Fig. 6B). However, even though control and low PQ treatment worms had no statistically significant difference in total reproduction, they did have a distinct difference in relative fitness (Wilcoxon rank sum, $\alpha = 0.05$). Finally, unlike the 25 °C assay, variance in fitness contracted with increasing oxidative stress (Levene's test, $F = 9.44$, $p < 0.001$, Fig. 6B).

Correlations Among Life History Traits

I compared life history traits with one another to determine whether predicted life history tradeoffs (negative correlations among fitness-related traits) would be revealed as stressful conditions increased.

25 °C

Figures 7 and 8 show average Spearman's pairwise rank correlations (ρ) between life history traits for each oxidative stress level. Figure 7 reports correlations between the traits that are most informative with respect to testing the central hypothesis that oxidative stress mediates life history tradeoffs. Figure 8 reports associations between traits that are necessarily correlated with one another to some degree due to overlapping measurement (e.g., total reproduction and relative fitness) but which nonetheless reveal

important information about the consequences of oxidative stress. Although many correlations tended to be negative in sign, there were very few statistically significant correlations ($\alpha = 0.05$) of either sign at any treatment level (see also Table 4). In fact, only two correlations were statistically distinguishable from zero. The first was between lifespan and late reproduction (see Methods) at the low PQ treatment level (Fig. 7). The second was a positive correlation between lifespan and early reproduction in the high-PQ treated cohort. In this trial, most of the significant correlations exist between the less informative pairs of traits (Fig. 8). This figure does, however, highlight the changing contributions of different life history components to relative fitness with increasing oxidative stress. For example, the correlation between relative fitness and lifespan, while zero in the other treatments, becomes significant and positive in high PQ treated worms ($\rho = 0.32$, $p = 0.022$).

20 °C

Figures 9 and 10 show average Spearman's pairwise rank correlations (ρ) between life history traits of worms grown at 20 °C and fed UV-killed bacteria and experiencing increasing levels of oxidative stress. In stark contrast to the previous assay, I observed a trend for life history correlations to become increasingly positive and statistically significant in the high PQ treatment cohort compared to the other treatment levels (Fig. 9). For this high-stress treatment, lifespan was significantly positively correlated with early reproduction ($\rho = 0.68$, $p = 0.0003$) and with total reproduction ($\rho = 0.58$, $p = 0.004$). Early and late reproduction are also correlated positively with each other ($\rho = 0.63$, $p = 0.0021$). The only other statistically significant correlations appeared

in the low PQ treatment between total reproduction and lifespan, which were positively related to one another ($\rho = 0.36$, $p = 0.02$) and between early and late reproduction, which were negatively correlated ($\rho = -0.35$, $p = 0.03$).

Correlations between relative fitness and fitness-related life history traits revealed the expected positive associations (Fig. 10, Table 5). These findings are at least qualitatively similar to those of the 25 °C assay apart from the negative correlation between lifespan and relative fitness observed for the control treatment (Fig. 10). Additionally, late reproduction appears to be a stronger contributor to overall fitness in this assay compared to the 25 °C assay (Figs. 8 and 10).

In Vivo ROS Levels

In order to further understand how effectively these nematodes controlled ROS levels and how this affected the pattern of life history traits seen in previous assays, I measured *in vivo* ROS levels.

25 °C

To assess relative levels of ROS among oxidative stress treatments, we subjected worms to MitoSOX Red (Invitrogen) in their respective PQ treatments at either 24 or 48 hours to understand whether the pattern of ROS levels would be affected by age (see Methods). Dingley et al. (2010) measured relative fluorescence units (RFU) in nematodes exposed to a 24 hr 1 mM PQ treatment, and found essentially equivalent RFU values for control and PQ treated worms. We expected to see similar patterns of RFU levels in our worms which were treated with much lower concentrations of PQ. Analysis of

pharyngeal bulb fluorescence after 24 hours of PQ treatment revealed significant variation among oxidative stress treatment levels (ANOVA, $F = 22.3$, $p < 0.0001$) and statistically significant differences between every pair of treatments (Tukey HSD, $\alpha = 0.05$) (Fig. 11A, filled symbols). ROS levels show an approximately linear decline from control levels to the low and medium PQ regimes and then increase slightly in the high PQ treatment level (control: 2.68 ± 0.13 ; low: 2.43 ± 0.18 ; medium: 2.10 ± 0.20 ; high: 2.27 ± 0.21).

In contrast to the above pattern, worms imaged at 48 hours after L1 arrest (i.e., young adults in the control treatment) showed a different pattern of ROS level with increasing oxidative stress (Fig. 11A, open symbols). Fluorescence levels in control worms were higher relative to the control cohort in the 24 hr trial, although not significantly so ($t = 0.53$, $p = 0.699$). ROS levels were higher at 48 hours than at 24 hours in both the low and medium PQ treatments, especially for the medium PQ treatment. Conversely, ROS levels were lower at 48 hours in the high PQ treated worms than for their 24-hour trial counterparts (2.27 ± 0.04 vs. 2.208 ± 0.04 ; Wilcoxon/ Kruskal-Wallis rank sum $Z = -3.90$, $p < 0.0001$) (Fig. 11).

20 °C

I also quantified relative ROS levels (RFU) at 20 °C in worms fed live *E. coli* (Fig. 11B). Unfortunately, a direct comparison cannot be drawn between ROS levels at 20 °C and 25 °C as the filter set and lenses were changed in between assays; the trends relative to controls within each assay may, however, be confidently compared. For this assay, I captured fluorescent images of worms at 68 hours in an attempt to measure ROS

at developmental stages similar to those in the 25 °C assay; i.e., at 48 hours (see Methods). The 68 hour worms, a time at which the nematodes in the medium PQ treatment had (on average) developed into young adults, the trend in ROS values for 20 °C worms (Fig. 11B) is similar to 25 °C worms at 48 hours (Fig. 11A, squares), although ROS levels in animals at the medium treatment are similar to the low treatment cohort and no longer higher than control worms.

Discussion

This thesis aimed to understand the physiological basis of life history tradeoffs by characterizing the effects of physiological stress on the interplay among fitness-related life history traits. I used laboratory studies with the model soil nematode, *Caenorhabditis elegans*, wherein animals were exposed to oxidative stress in the form of paraquat or a combination of oxidative and mild thermal stress. For animals in each treatment, I performed basic life tables to estimate fitness components and conducted fluorescence confocal microscopy to measure *in vivo* ROS levels. My central hypothesis was that increased levels of physiological stress would unmask life history tradeoffs predicted by classical theory.

Nematode Oxidative and Thermal Stress Experiments

There is a rich literature on oxidative stress-resistance in wildtype and mutant *C. elegans* genotypes. These assays almost always utilize acute and extreme oxidative insult while measuring the survival of focal nematodes in terms of hours or by assessing survival percentage after a few days (47,49,50,52,53,57,64,75–82). These studies

commonly use paraquat as the source of ROS, but some have used other ROS generators such as juglone (53,80), H₂O₂ (82), or sodium arsenite (81). When lower concentrations of prooxidants are used in lifespan assays, they are generally used in conjunction with 5-fluoro-2'-deoxyuridine (FUdR) (50,63), which prevents mitotic cell division by inhibiting DNA synthesis. When applied to adult nematodes, FUdR simplifies lifespan assays by preventing oogenesis and thereby preventing reproduction (51). However, FUdR has also been shown to cause additional lifespan extension in two long-lived *C. elegans* mutants (83,84), suggesting that the effects of this compound may not be fully understood. In any case, the trend of FUdR use has made assessment of fitness modification by oxidative or other stressors a rare occurrence indeed. Further, the use of FUdR encourages more brute-force attempts to assess resistance to unrealistic levels of oxidative stress. It is unlikely that acute oxidative stress is a common threat to *C. elegans*, which generally inhabits UV-free and hypoxic soil conditions (85). Since my research goal was to record how life history traits respond to biologically relevant levels of oxidative stress, I reasoned that a chronic exposure assay would be preferable.

To impose mild thermal stress on *C. elegans* populations, I chose to culture nematodes at 25 °C. Although thermal regime appears to be an issue of lab preference, much previous *C. elegans* research has been performed at 20 °C (17,49,50,52,75,76,80,86–89), which is the accepted thermal optimum for N2 *C. elegans* in evolutionary studies (67). 25 °C ± 1C is also frequently used (47,53,62–65,75,78,90,91), perhaps primarily as a means to shorten the length of *C. elegans* life cycles. Culturing worms at higher temperatures reduces lifespan and mean total

fecundity (22) and reduces maturation time (92) presumably through processes including a general increase in enzyme activities (93).

Reproductive Traits

The level of oxidative stress treatments at 25 °C using live *E. coli* as a food source produced significant variation in reproductive traits (Fig. 1A; Table 1). Surprisingly a hormetic increase in total reproduction was found in worms exposed to low level oxidative insult relative to controls (Fig. 1A). This effect is interesting in light of the fact that self-reproductive capacity in these worms is known to be sperm limited (94). Total reproductive output declined slightly in medium PQ treated worms and further declined to control levels in high PQ treatment worms. A shift in the reproductive period effectively increased the generation time of the nematodes in the high PQ treatment (Fig. 3), however, and resulted in a significant reduction in their relative fitness when compared to controls (Fig. 6A). A previous study established that paraquat treatment caused a delay in maturation rate resulting in a shift in reproductive schedule without a reduction in fecundity (95), consistent with my findings. I hypothesize that this reproductive delay was caused by somatic maintenance, specifically the increased allocation of resources to oxidative stress response.

Compared to the 25 °C assay, reproductive output, timing, and relative fitness were more profoundly affected by paraquat treatment at 20 °C using UV-killed *E. coli* as a food source. Total reproductive output showed a linear decline with increasing oxidative insult (Fig. 1B, Table 2). Combined with the severe delay in reproductive schedule that accompanied increasing oxidative stress (Fig. 2B), this reduced

reproduction led to a sharp decline in relative fitness with increasing PQ treatment (Fig. 6B). Further, phenotypic variation among PQ treatment groups was elevated in worms fed UV-killed *E. coli* compared to those on the live *E. coli* diet; see F-ratios in Table 5 (UV-killed) versus Table 4 (live). The more pronounced effects of oxidative stress observed in this experiment may be from reduced SOD levels and/or dampened innate immune responses due to nematodes being fed a diet of UV-killed bacteria (see Materials and Methods). In support of the first idea, Jonassen *et al.*, (88) found that *clk-1* mutants, which are unable to synthesize coenzyme Q (Q₉), had arrested development unless their diet was supplemented with an alternative quinone, Q₈, from OP50 *E. coli*. In addition to functioning as coenzymes within the mitochondrial ETC, quinones also complement the regular array of antioxidant molecules *in vivo*, functioning as lipid soluble free radical sinks in the cell (88). It is not known which other antioxidant molecules *C. elegans* obtains from its diet, but it is reasonable to suppose that nematodes in the 25 °C/live *E. coli* experiment were acquiring an antioxidant buffer from their living bacterial food source whose own antioxidant systems were likely upregulated in response to the PQ treatment (96). In contrast, UV-killed *E. coli* are not producing proteins or metabolites that might assist antioxidant capacity in *C. elegans* exposed to paraquat. The current study is thus somewhat hobbled by the lack of a life history assay at 20 °C using live bacteria, which would allow a direct assessment of the effects of food source on oxidative stress resistance in *C. elegans* without the possible confounding effects of temperature (see Chapter 3). However, a comparison of the maturation rate assays using live bacteria at both 20 °C and 25 °C (Fig. 3) and preliminary studies (data not shown) supported the idea that food source accounted for much of difference observed between assays in

reproductive output in PQ-exposed worms. Specifically, control worms eating UV-killed *E. coli* developed much more slowly than those given live food, and a study at 20 °C using live *E. coli* has shown that reproduction at this temperature is resistant to perturbation within the levels of oxidative stress tested here.

The shifts in reproductive timing observed with increasing PQ treatment observed in each assay (Fig. 3) may constitute a life history tradeoff within the framework of somatic maintenance prioritization over reproduction, because delaying the time to reproductive maturation can result in a major disadvantage for nematodes in terms of reproductive fitness (c.f., Hodgkin and Barnes 1991). Alternatively, delayed reproduction in times of stress could provide a fitness advantage if it allowed an organism to “wait out” the stressful conditions and produce offspring under more favorable conditions - even if the delay were accompanied by reduced total fecundity (98). Such a scenario is unlikely to explain the observations of this study since nematodes are not likely to encounter considerable levels of oxidative stress, fluctuating or otherwise, in nature. The pattern of delay in maturation rate observed with increasing levels of paraquat was not obscured by an interacting effect of temperature (Fig. 3; Table 3). As visualized in Fig. 3, PQ treatment and temperature each had significant effects on maturation rate, the latter of which produced an ~20 hr delay in maturation rate at 20 °C as compared to 25 °C; however, these factors did not interact in their effects on reproductive delay (Fig. 3).

Lifespan

The mechanism(s) driving senescent decline, a universally documented condition among eukaryotes, is not currently known. Several factors like the accumulation of age-correlated molecular aggregates (lipufuscin), decline in muscle mass, increase in genetic and protein damage, and loss of reproductive capability late in life have been linked to oxidative stress in *C. elegans* and have been used as evidence toward an oxidative stress theory of aging (43). In support of the oxidative stress theory of aging, resistance to prooxidants (along with resistance to temperature, UV irradiation, and hyperoxia) is a hallmark of long-lived mutants (87) possibly through upregulation of antioxidant defenses (76), although the biological relevance to certain of these stressors is an open question (85). I hypothesized that average lifespan would be negatively correlated with the level of oxidative insult in agreement with the oxidative stress theory of aging. Since ROS are known to damage to all macromolecules, lifespan should be one of the most obviously affected life history traits as this damage should accumulate with age (11). However, recent research suggests that an oxidative stress theory of aging may have been either an oversimplification or a case of causation attributed to strong correlation (75,99–101). For example, Doonan, *et al* (49) found that RNAi knockdown of *sod-2*, a *C. elegans* gene coding for a cellular superoxide dismutase thought to provide a vital antioxidant function, resulted in higher levels of molecular oxidative damage, but no change in lifespan. In addition, I found no differences in nematode lifespan among PQ treatments in either life history trial (Fig. 4A and 4B), and variance in lifespan did not change significantly for either life history assay (Tables 4 and 5; Levene's test, $\alpha = 0.05$) further chipping away at the oxidative stress theory of aging. These results are especially

surprising when considering the life history assay at 20 °C using UV-killed *E. coli*. PQ treatment caused substantial reductions in reproduction and relative fitness relative to control worms, yet lifespan was not negatively impacted with increasing oxidative insult. Reducing temperature increased lifespan, as expected, but had no interaction effect with oxidative treatment (Table 3, Fig. 5).

Life History Tradeoffs

Life history theory predicts that reproductive output and schedule should be significant determinants of lifespan (4,18) and several studies have confirmed so-called ‘costs of reproduction’ (20,25,102). For example, increased early reproduction should cause a reduction in lifespan owing to a genetic or physiological constraint. By the same token, if reproduction declines, there should be a positive response in the lifespan (98). Accordingly, long-lived age mutants in *C. elegans* are known to have delayed and reduced fecundity (57,85,90,103,104). Within this context, I calculated the correlations among major life history traits in both assays. In contrast to the predicted tradeoffs, I found few significant correlations between lifespan and reproductive traits including total, early and late reproductive output (see Methods) for the 25 °C assay (Table 4). Surprisingly, lifespan was negatively correlated with late reproduction in low PQ treated worms (Table 4, Fig. 7); lifespan and early reproduction were positively related in high PQ treated worms (Fig. 7). Both of these observations contrast with my initial predictions. Correlations between reproduction traits fitness were positive as expected due to the overlapping nature of those measurements. At 20 °C, even stronger correlations were revealed, but they too were against predictions (Table 5, Figs. 9 and

10). In fact, at 20 °C the worms exposed to the highest level of PQ had strongly positive correlations between reproductive output and lifespan. It is apparent that the simple model of a fixed total energy budget being divided between reproduction and somatic maintenance (c.f., Stearns, 1989) may be insufficient to describe the true nature of life history tradeoffs in this system.

The source of the strongly positive life history trait correlations observed in this study assays is unknown but could result from short-term evolutionary processes that occurred in our experiments. Paraquat should produce stochastic macromolecular damage through its production of superoxide anions (12,55,105,106) and may therefore have generated genetic variation in our otherwise isogenic *C. elegans* populations. Since paraquat was applied to large populations of nematodes, genetic variants best able to endure the PQ treatments could have been enriched via selection within each starting population. Among these survivors, lifespan and reproduction were positively related. A direct test of this hypothesis would necessitate a study of the effects of the paraquat concentrations applied here on genomic stability.

In Vivo ROS Levels

I employed a method of *in vivo* ROS measurement established previously (72, 73). Previous results have shown ROS levels measured by fluorescent microscopy are similar to those measured in electron spin resonance assays (107). Additionally, ROS levels obtained via fluorescent microscopy from natural isolates of the congeneric nematode, *C. briggsae*, have been shown to correlate strongly with levels of oxidative DNA damage (J. Joyner Matos and S. Estes, unpubl. data). In the 25 °C assay, ROS

measurements in larval worms decline with PQ concentration and then increased slightly at the highest PQ treatment level – though not significantly beyond low PQ or control levels (Fig. 11A). This result indicates that larval worms are quite capable of suppressing ROS proliferation when treated with paraquat; the uptick in ROS at the highest PQ treatment level may indicate that a threshold to their antioxidant capacity is being approached. In accord with previous results showing that adult (or ‘mixed stage’) worms have reduced antioxidant capacity (60), low and medium PQ treatment cohorts had higher ROS levels at 48 hours (i.e., adult stage worms as determined via maturation rate assays) as compared to 24 hours (larval stage) (Fig. 11A). Worms tested after 48 hours in the high PQ level exhibited the lowest level of ROS, which was also lower than its larval stage counterparts (Fig. 11A). Maturation rate as modulated by PQ increase (Fig. 3) may provide an explanation for these findings; nematodes at the highest PQ treatment level are the slowest to mature to adulthood (high PQ, 51.0 ± 4.3 hrs; control, 42.4 ± 2.8 hrs) and might indicate that these worms are still in a larval stage and producing greater amounts of antioxidants to deal with the ROS generated by paraquat. This age difference as modulated by paraquat dosage might also then explain the difference in ROS measures between the medium and high PQ treated worms at 48 hours. Nematodes in the medium PQ treatment exhibited control levels of ROS (i.e., increased compared to that of high PQ treated worms) suggesting that antioxidant production may be reduced as these worms enter adulthood during this time.

ROS measurements of *C. elegans* raised at 20 °C and at a developmental time point analogous to 48 hrs in the 25 °C assay (taking into account the increased

developmental time at 20 °C, Fig. 3) revealed a similar pattern of ROS level (compare Fig. 11A and 11B). Most notable is that the lowest ROS level was detected in the high PQ treated worms. The medium PQ cohort had higher levels of ROS relative to the high treatment as was the case at 48 hours, which provides further support for an age-dependent modulation of antioxidant production during chronic stress. Here, however, ROS levels of medium PQ worms were lower than in control worms. This may reflect a reduction in the overall rate of endogenous ROS production at the lower temperature, but this was not tested.

While there were no quantifiable tradeoffs in the life history characters I tracked, these ROS data are strongly indicative of a tradeoff between reproductive fitness and antioxidant production. A previous study confirmed the upregulation of the antioxidant coding gene *sod-3* in larval worms after having been exposed to paraquat (60). High treatment worms in this study had significantly lower ROS levels (Fig. 11), suggesting the upregulation of antioxidants with fitness levels reduced by over 40% (Fig 6A), primarily through a delay in maturation (Fig 3). This suspected tradeoff was even more pronounced when the diet of *C. elegans* was changed to UV-killed bacteria (presumably reducing bacterial antioxidants available to the nematodes). Worms subjected to high levels of oxidative stress experienced a 13-fold reduction in relative fitness when compared to the control cohort.

Summary

This study provides support for the idea that somatic maintenance has priority over reproduction during periods of chronic oxidative and thermal stress. However, life

history tradeoffs are not consistently manifested under stress in the fitness-related traits analyzed here. I have shown that reproductive traits are strongly negatively affected by oxidative stress, but that lifespan is entirely resilient to paraquat-derived oxidative. The latter finding provides evidence against the oxidative stress theory of aging. Further support for the resource allocation hypothesis comes from the finding that reproductive delays caused by oxidative stress treatment are accompanied by reduced ROS presumably through upregulation of antioxidant proteins. Finally, I provide evidence that the ability of *C. elegans* to withstand oxidative stress is related to its food source; *C. elegans* on a live-bacteria diet can mount a more effective response to ROS, through bacteria-derived metabolites and/or antioxidant molecules.

Tables and Figures

Table 1. Life History Traits at 25 °C on Live *E. coli*

| <i>Trait</i> | <i>PQ</i> | <i>Mean (SEM)</i> | <i>n</i> | <i>F-ratio</i> | <i>p-value</i> |
|-------------------------------|-----------|-------------------|----------|----------------|----------------|
| Total Reproduction | Control | 176.52 (6.14) | 56 | 5.4217 | 0.001 |
| | Low | 205.72 (6.25) | 54 | | |
| | Medium | 198.91 (6.31) | 53 | | |
| | High | 178.71 (6.37) | 52 | | |
| Early Reproduction | Control | 143.80 (4.89) | 56 | 3.8095 | 0.01 |
| | Low | 160.17 (4.98) | 54 | | |
| | Medium | 160.59 (5.03) | 53 | | |
| | High | 166.19 (5.08) | 52 | | |
| Late Reproduction | Control | 33.31 (3.51) | 55 | 17.2848 | <0.0001 |
| | Low | 47.92 (3.64) | 51 | | |
| | Medium | 39.06 (3.61) | 52 | | |
| | High | 12.52 (3.61) | 52 | | |
| Relative Fitness (ω) | Control | 1.02 (0.03) | 56 | 39.7605 | <0.0001 |
| | Low | 1.12 (0.03) | 54 | | |
| | Medium | 1.00 (0.03) | 53 | | |
| | High | 0.66 (0.03) | 52 | | |
| Lifespan | Control | 12.71 (0.53) | 56 | 0.4910 | 0.69 |
| | Low | 13.19 (0.54) | 54 | | |
| | Medium | 13.19 (0.55) | 53 | | |
| | High | 12.40 (0.55) | 52 | | |

ANOVA tables for life history traits among PQ treatment levels (see methods for detailed treatment information) for nematodes at 25 °C on live OP50-1 *E. coli*. Standard error (SEM) is reported in parentheses following the respective mean for each trait within a treatment level. Sample size (n) is listed in addition to F-ratio. P-value considered significant at $\alpha = 0.05$. Degrees of freedom for all traits is 3.

Table 2. Life History Traits at 20 °C on UV-killed *E. coli*

| <i>Trait</i> | <i>PQ</i> | <i>Mean (SEM)</i> | <i>n</i> | <i>F-ratio</i> | <i>p-value</i> |
|-------------------------------|-----------|-------------------|----------|----------------|----------------|
| Total Reproduction | Control | 223.87 (7.32) | 39 | 65.7856 | <0.0001 |
| | Low | 209.37 (7.42) | 38 | | |
| | Medium | 148.32 (7.52) | 37 | | |
| | High | 71.04 (9.54) | 23 | | |
| Early Reproduction | Control | 75.08 (4.37) | 39 | 19.0686 | <0.0001 |
| | Low | 73.13 (4.43) | 38 | | |
| | Medium | 53.57 (4.49) | 37 | | |
| | High | 26.57 (5.69) | 23 | | |
| Late Reproduction | Control | 148.80 (5.77) | 39 | 43.4533 | <0.0001 |
| | Low | 136.24 (5.84) | 38 | | |
| | Medium | 94.76 (5.92) | 37 | | |
| | High | 48.71 (7.86) | 21 | | |
| Relative Fitness (ω) | Control | 1.05 (0.04) | 39 | 77.8383 | <0.0001 |
| | Low | 0.85 (0.04) | 38 | | |
| | Medium | 0.39 (0.05) | 37 | | |
| | High | 0.08 (0.06) | 23 | | |
| Lifespan | Control | 23.62 (1.09) | 39 | 0.6875 | 0.56 |
| | Low | 22.13 (1.11) | 38 | | |
| | Medium | 24.30 (1.12) | 37 | | |
| | High | 22.91 (1.42) | 23 | | |

ANOVA tables for life history traits among PQ treatment levels (see methods for detailed treatment information) for nematodes at 20 °C kept on UV-killed *E. coli*. Standard error (SEM) is reported in parentheses following the respective mean for each trait within a treatment level. Sample size (n) is listed in addition to F-ratio. P-value considered significant at $\alpha = 0.05$. Degrees of freedom for all traits is 3.

Table 3. Results of ANOVA Model Analysis for Maturation Assays

| <i>Source of Variation</i> | <i>df</i> | <i>F-ratio</i> | <i>p-value</i> |
|----------------------------|-----------|----------------|----------------|
| Temperature | 1 | 2695.9 | <0.0001 |
| PQ Treatment | 3 | 110.8 | <0.0001 |
| Temperature × PQ Treatment | 3 | 0.95 | 0.42 |

Summary of two-way ANOVA for maturation rate assays conducted at 20 °C and 25 °C. Variance in maturation rate depends on temperature regime and PQ level but not on the interaction of these factors. Both assays were performed using live OP50-1 *E. coli*.

Table 4. Spearman Pairwise Correlations and Individual Trait Variances at 25 °C on live *E. coli*

| | | Early | Late | Total | Fitness | Lifespan |
|---------|----------|-------|-------|-------------|-------------|--------------|
| Control | Early | 28.94 | -0.03 | 0.71 | 0.88 | 0.01 |
| | Late | | 20.50 | 0.64 | 0.10 | -0.12 |
| | Total | | | 35.40 | 0.71 | -0.08 |
| | Fitness | | | | 0.20 | -0.01 |
| | Lifespan | | | | | 4.14 |
| Low | Early | 27.67 | 0.02 | 0.74 | 0.91 | -0.08 |
| | Late | | 27.09 | 0.74 | 0.16 | -0.31 |
| | Total | | | 43.79 | 0.66 | -0.12 |
| | Fitness | | | | 0.19 | -0.10 |
| | Lifespan | | | | | 4.47 |
| Medium | Early | 41.12 | 0.03 | 0.64 | 0.70 | -0.11 |
| | Late | | 35.87 | 0.70 | 0.49 | -0.13 |
| | Total | | | 52.92 | 0.78 | -0.18 |
| | Fitness | | | | 0.30 | -0.22 |
| | Lifespan | | | | | 3.93 |
| High | Early | 46.09 | 0.04 | 0.84 | 0.82 | 0.28 |
| | Late | | 16.76 | 0.44 | -0.17 | -0.05 |
| | Total | | | 50.39 | 0.62 | 0.15 |
| | Fitness | | | | 0.21 | 0.32 |
| | Lifespan | | | | | 3.29 |

PQ Treatment groupings on left, vertical. Individual trait variance given in diagonal (gray) for early, late and total reproduction, and fitness and lifespan. Statistically significant correlations are in bold ($\alpha = 0.05$). Data taken from life history assay at 25 °C using live *E. coli*.

Table 5. Spearman Pairwise Correlations and Individual Trait Variances at 20 °C on UV-killed *E. coli*

| | | Early | Late | Total | Fitness | Lifespan |
|---------|----------|-------|--------------|-------------|-------------|--------------|
| Control | Early | 27.18 | -2.6E-03 | 0.61 | 0.64 | -0.24 |
| | Late | | 31.90 | 0.72 | 0.39 | -0.01 |
| | Total | | | 39.50 | 0.63 | -0.02 |
| | Fitness | | | | 0.36 | -0.36 |
| | Lifespan | | | | | 6.74 |
| Low | Early | 31.87 | -0.35 | 0.39 | 0.38 | 0.07 |
| | Late | | 41.79 | 0.65 | 0.19 | 0.29 |
| | Total | | | 47.59 | 0.43 | 0.36 |
| | Fitness | | | | 0.31 | 0.07 |
| | Lifespan | | | | | 7.24 |
| Medium | Early | 24.51 | 0.11 | 0.62 | 0.86 | -0.03 |
| | Late | | 36.13 | 0.80 | 0.32 | -0.10 |
| | Total | | | 46.46 | 0.71 | -0.03 |
| | Fitness | | | | 0.20 | -0.13 |
| | Lifespan | | | | | 6.84 |
| High | Early | 23.14 | 0.63 | 0.88 | 0.90 | 0.70 |
| | Late | | 31.35 | 0.94 | 0.71 | 0.36 |
| | Total | | | 51.05 | 0.89 | 0.58 |
| | Fitness | | | | 0.09 | 0.68 |
| | Lifespan | | | | | 6.16 |

PQ Treatment groupings on left, vertical. Individual trait variance given in diagonal (gray) for early, late and total reproduction, and fitness and lifespan. Statistically significant correlations are in bold ($\alpha = 0.05$). Data taken from life history assay at 20 °C using UV-killed *E. coli*.

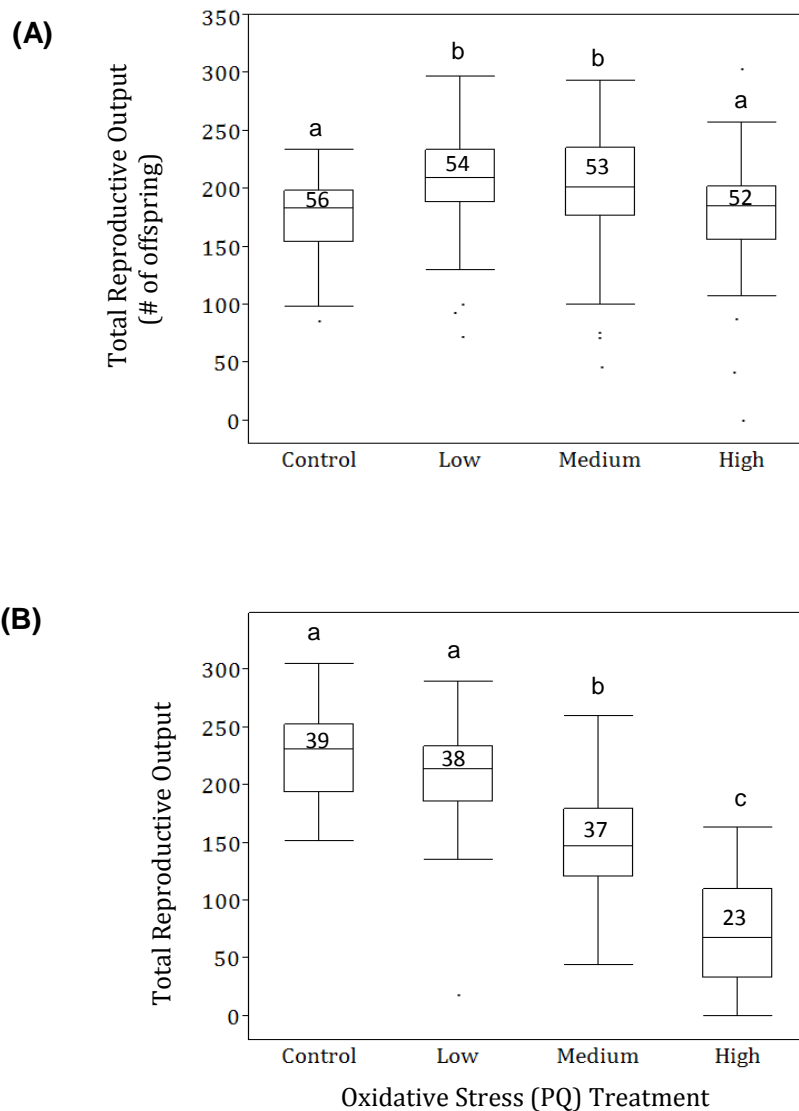


Figure 1. Total reproductive output in *C. elegans* is modified by temperature, oxidative stress, and diet. **(A)** Total fecundity increases at low and medium oxidative stress treatment levels and returns to control levels at the highest stress treatment level in worms fed live OP50-1 *E. coli* at 25 °C. **(B)** Total fecundity declines even with low levels of oxidative stress (NS) and continues to decline with increasing levels of stress in worms fed UV-killed *E. coli* at 20 °C. Letters above box plots group statistically identical means as indicated by pairwise Wilcoxon rank sum tests for non-parametric datasets ($\alpha = 0.05$) for panel A and Tukey-Kramer HSD, $\alpha = 0.05$. Horizontal lines within boxes indicate the median while the top and bottom of the boxes bound the upper and lower quartiles, respectively. Whiskers denote the minimum and maximum values; outliers shown as dots. Sample sizes are indicated by numbers within the boxes.

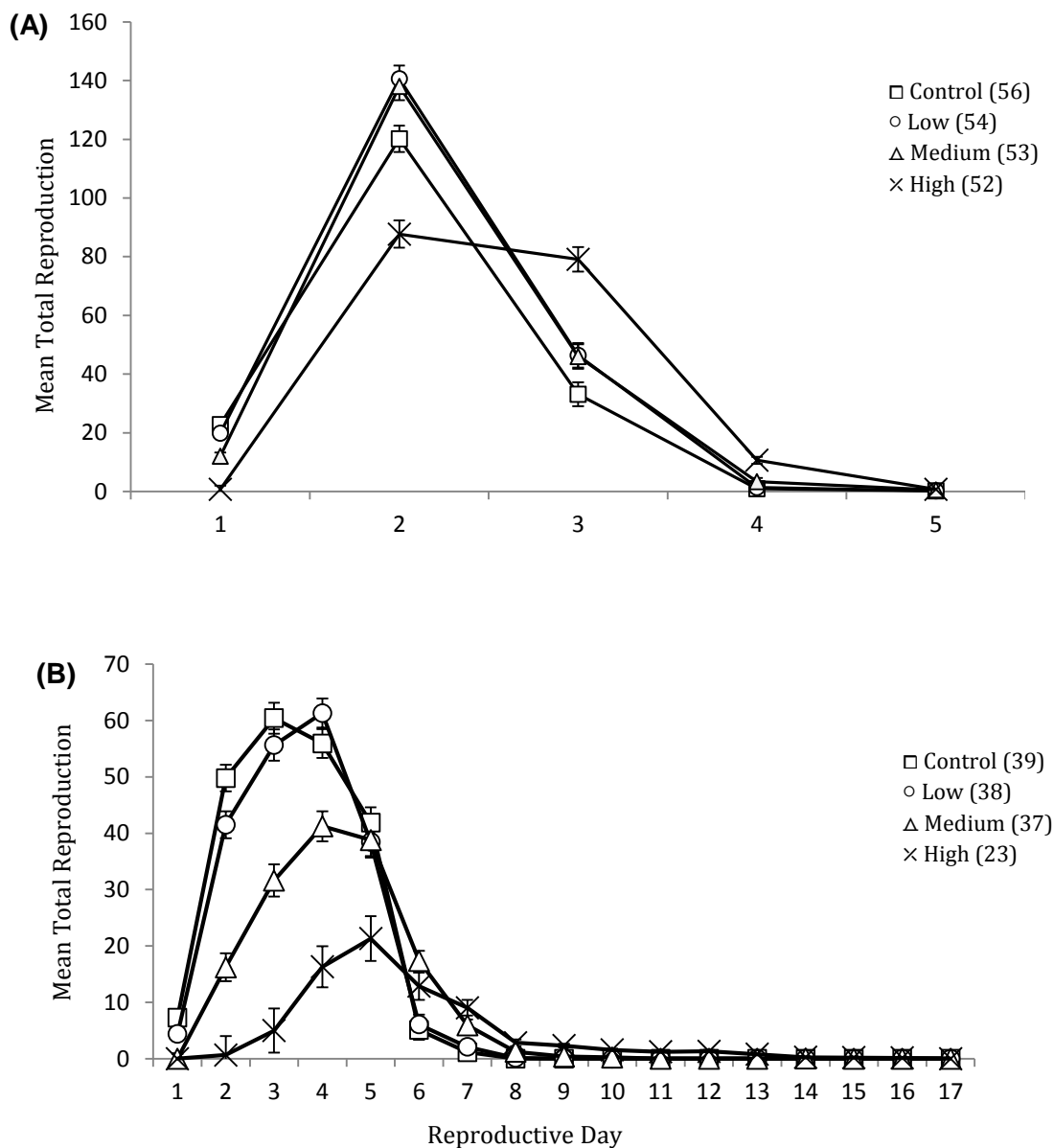


Figure 2. Reproductive schedules for each treatment level recorded as the mean number of surviving offspring per day of reproduction. Sample sizes listed parenthetically following legend entries. (A) Worms maintained at 25 °C with live OP50-1 *E. coli* have similar reproductive schedules under control, low and medium oxidative stress treatments, while worms experiencing the highest stress level have a slightly delayed reproductive schedule. (B) Worms maintained at 20 °C with UV-killed *E. coli* have lower overall reproductive output than worms experiencing 25 °C and live *E. coli* (note change in scale between A and B panels) and show a reproductive delay even at the lowest oxidative stress treatment level, a pattern that is amplified under medium and high stress levels. Further, reproductive schedules for worms in all stress treatments were prolonged as compared to those in (A), but particularly for those experiencing the highest stress level. Each symbol represents mean reproductive output for corresponding day. Error bars represent one standard error (SEM).

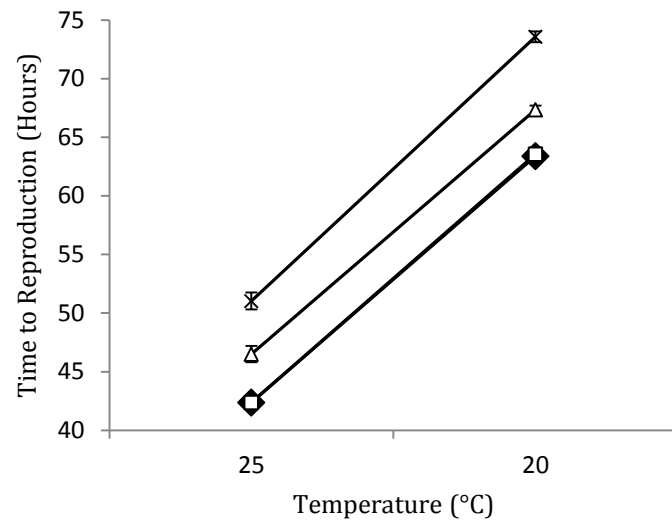


Figure 3. Time to reproduction determined by the hour of the first egg laid at control (squares) and three oxidative stress treatment levels (control= squares, low = diamonds, medium = triangles, high = x; See Methods for details on treatment scheme) for worms raised under two temperature regimes and fed live *E. coli*. (Note that control data points overlap those for the low oxidative stress treatment at both temperatures.)

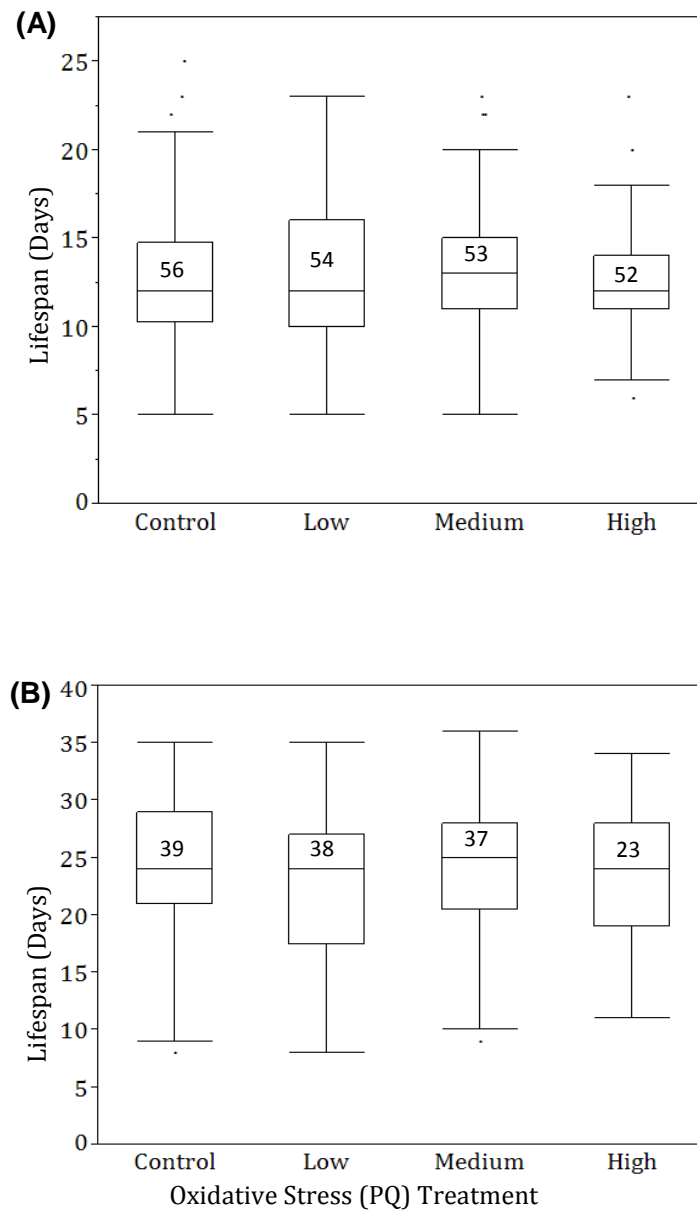


Figure 4. Mean lifespan as measured by total number of days lived beyond L1 stage arrest for worms experiencing (A) 25 °C and fed live OP50-1 *E. coli*. ANOVA testing indicated no significant variation among treatment means ($F = 0.49$, $p = 0.69$) (B) 20 °C and fed UV-killed *E. coli*. No significant differences in mean lifespan were detected among oxidative stress treatment levels (ANOVA $F = 0.69$, $p = 0.56$). However, worms in the second assay have increased mean lifespan as compared to the first (note change in scale between A and B panels; see Fig. 5 for details). Horizontal lines within boxes indicate the median while the top and bottom of the boxes bound the upper and lower quartiles, respectively. Whiskers denote the minimum and maximum values; outliers are shown as dots. Sample sizes are indicated by numbers within the boxes.

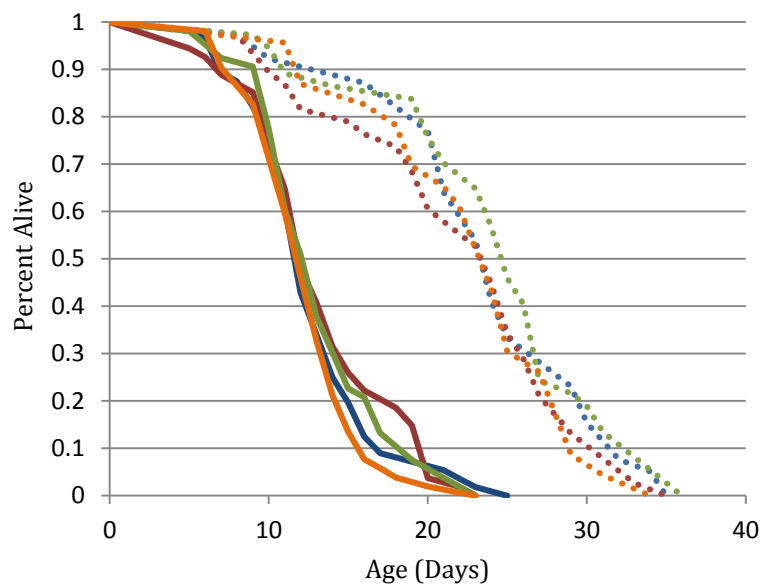


Figure 5. Survival curves for control worms raised at 25 °C on live *E. coli* (solid) or on UV-killed *E. coli* at 20 °C (dashed) . PQ treatments: Control (blue), Low (red), Medium (green), High (orange).

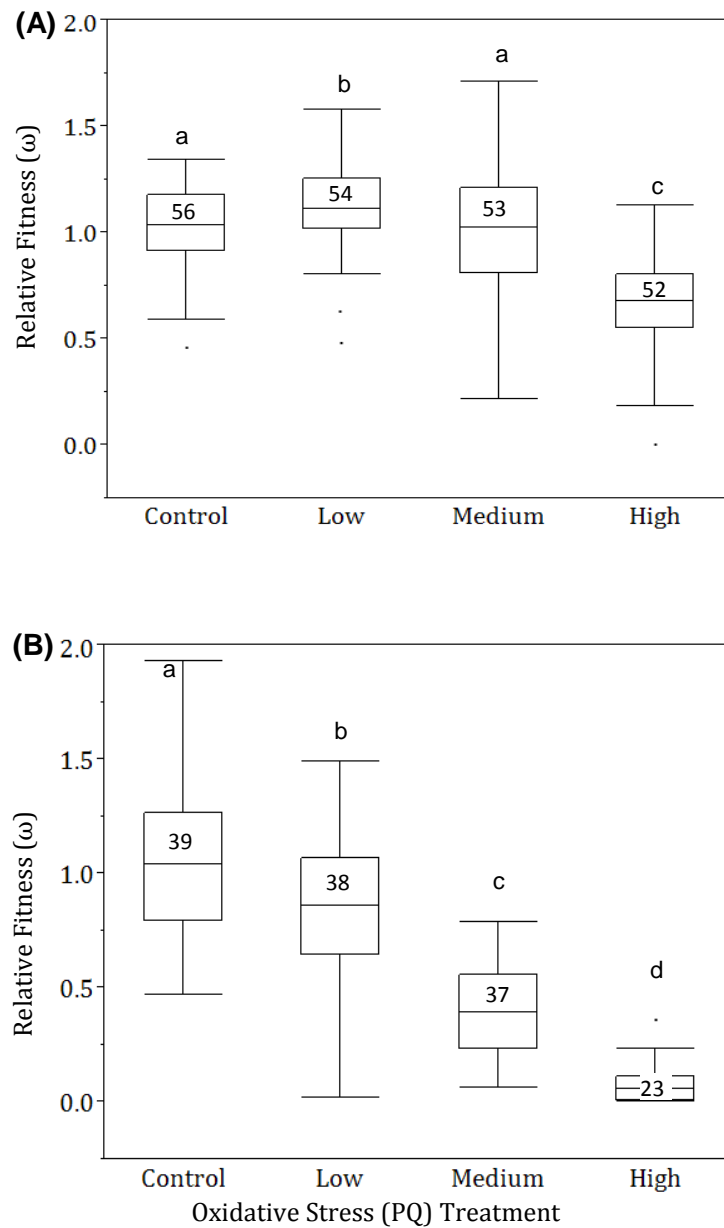


Figure 6. Relative fitness of worms experiencing **(A)** 25 °C and fed live *E. coli* and **(B)** 20 °C and fed UV-killed *E. coli*. Letters indicate statistically similar means as determined by pairwise Wilcoxon rank sum tests ($\alpha = 0.05$). Horizontal lines within boxes indicate the median while the top and bottom of the boxes bound the upper and lower quartiles, respectively. Whiskers denote the minimum and maximum values; outliers are shown as dots. Sample sizes are indicated by numbers within the boxes.

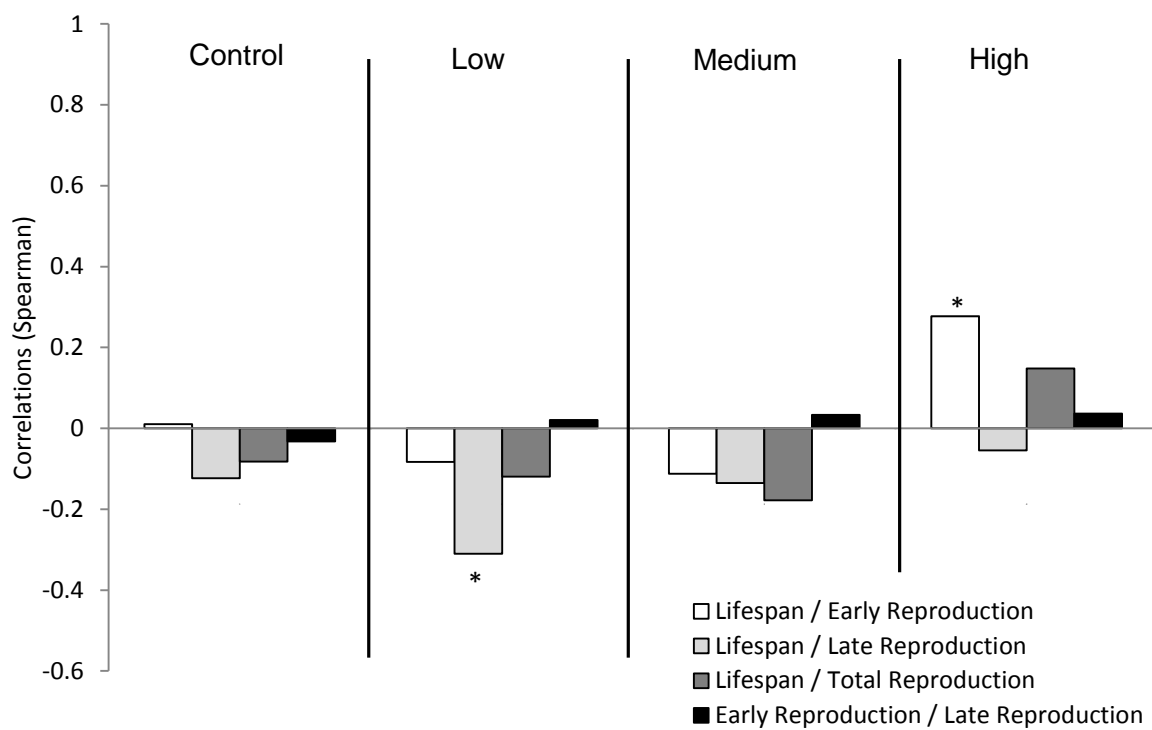


Figure 7. Spearman pairwise rank correlations for life history traits at each oxidative stress treatment level for worms grown at 25 °C and fed live OP50-1 *E. coli*. Statistical significance is represented by asterisks where * = $p < 0.05$.

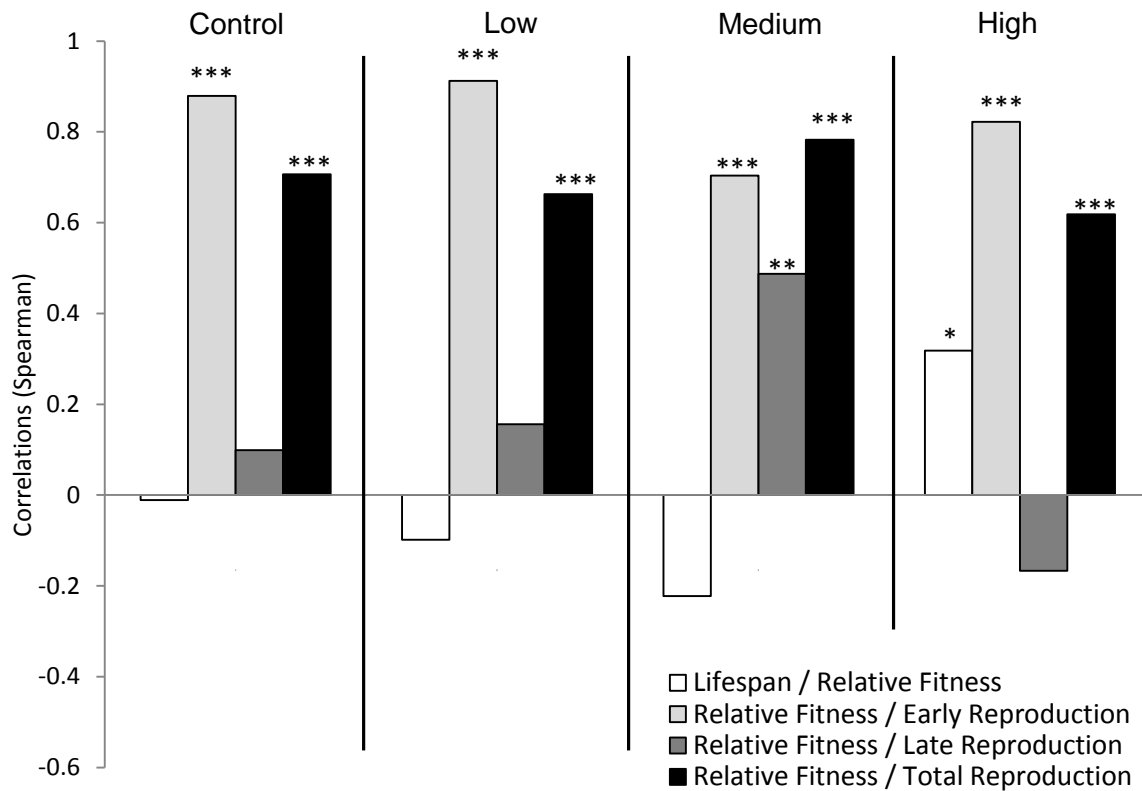


Figure 8. Spearman pairwise rank correlations for life history traits at each oxidative stress treatment level for worms grown at 25 °C and fed live OP50-1 *E. coli*. Statistical significance is represented by asterisks where * = $p < 0.05$, ** = $p < 0.001$ and *** = $p < 0.0001$.

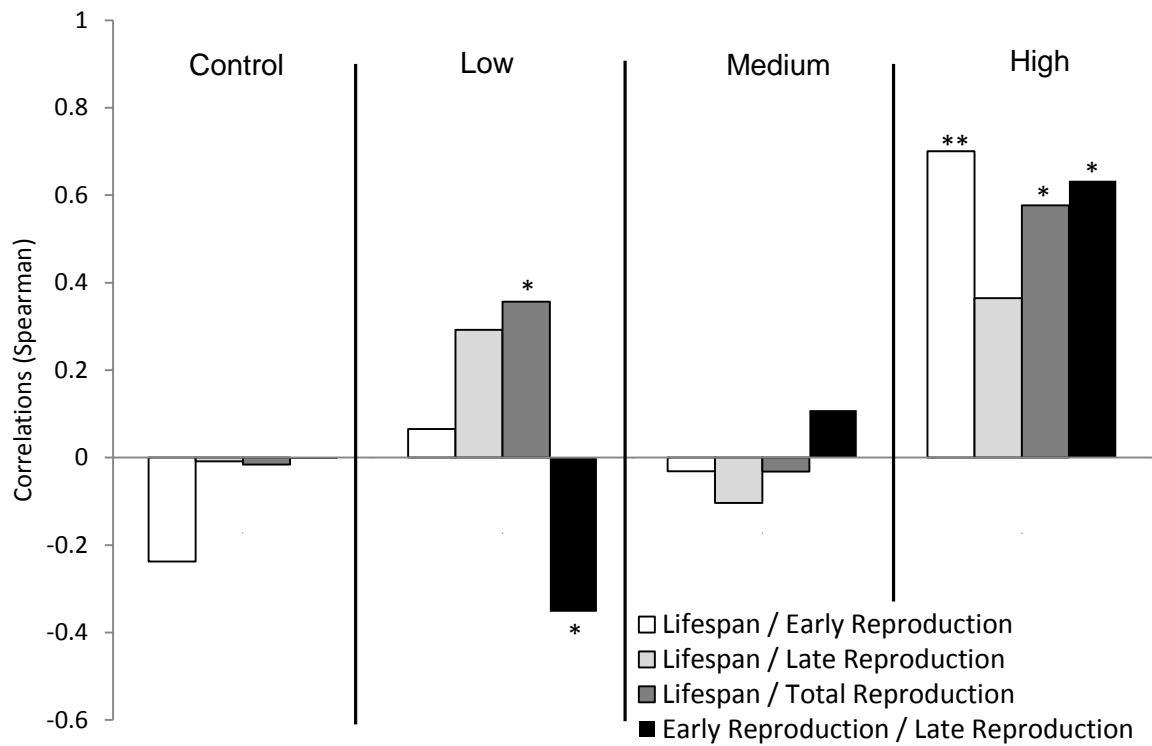


Figure 9. Spearman pairwise rank correlations for life history traits at each oxidative stress treatment level for worms grown at 20 °C and fed UV-killed OP50-1 *E. coli*. Statistical significance is represented by asterisks where * = $p < 0.05$, ** = $p < 0.001$.

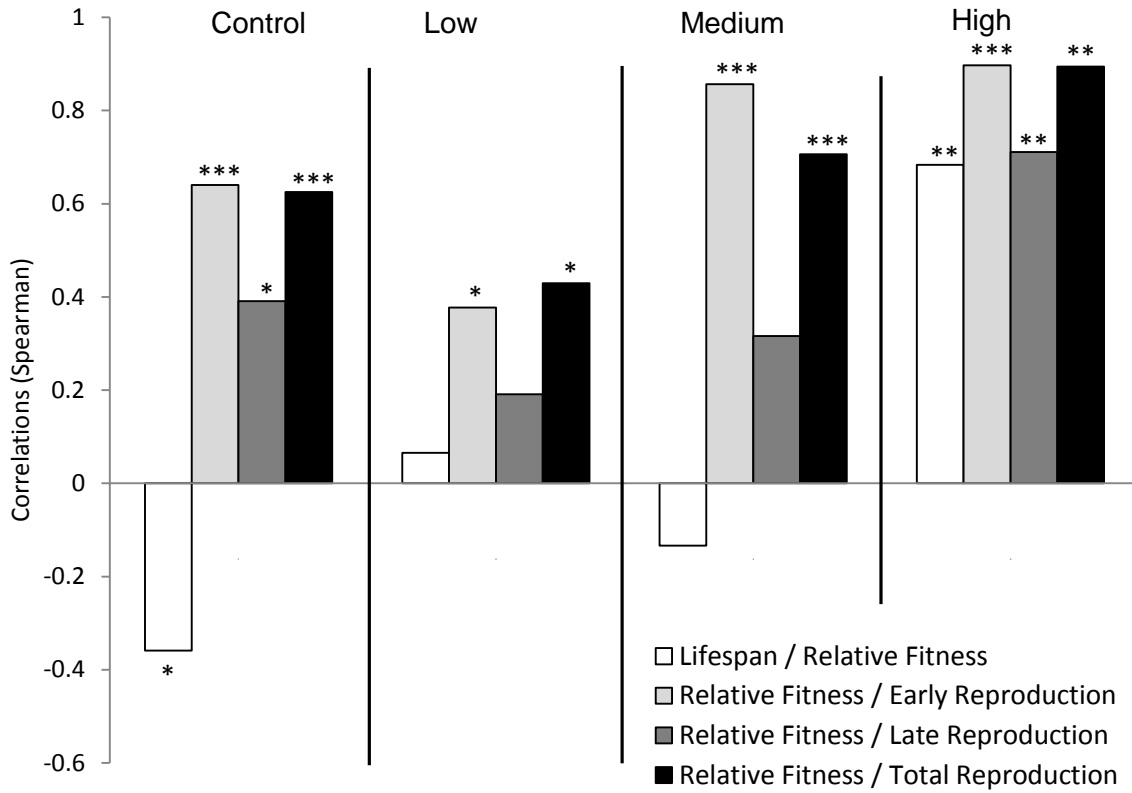


Figure 10. Spearman pairwise rank correlations for life history traits at each treatment level for worms grown at 20 °C and consuming UV-killed OP50-1. Statistical significance is given by number of asterisks as determined by p values; *: $p < 0.05$ & ≥ 0.001 , **: $p < 0.001$ & ≥ 0.0001 , and ***: $p < 0.0001$.

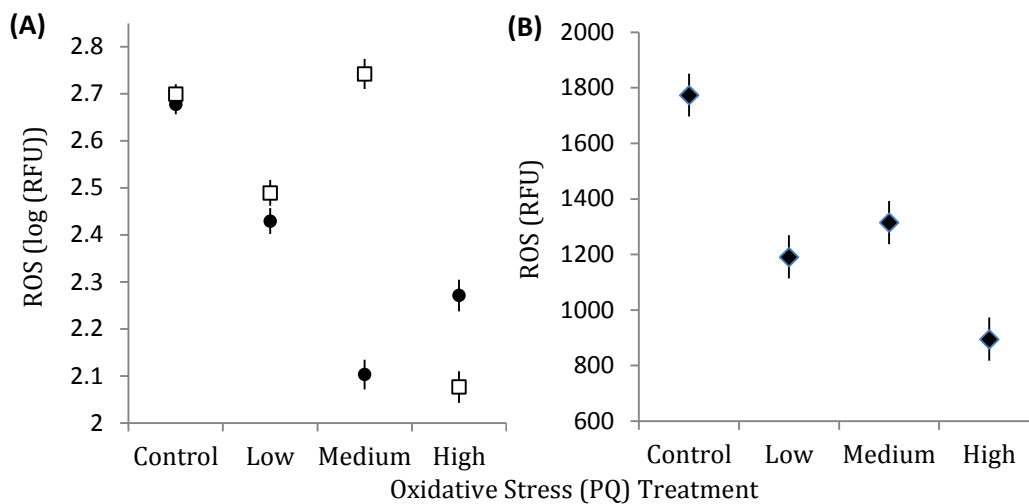


Figure 11. Reactive oxygen species (ROS) level measured as average maximum pixel intensity (RFU). (A) ROS levels in worms raised at 25 °C with live *E. coli*. Nematodes allowed to develop 24 hours (circles) and 48 hours (squares) after L1 arrest. Means log transformed to satisfy normality requirement of parametric tests. (B) ROS levels in worms allowed to develop for 68 hours (diamonds) raised at 20 °C with live *E. coli*. Compare trend in data points to those in panel A (squares). See Figure 3 for more information on delay in time to reproduction. Error bars are 1 SEM in both panels.

Chapter 3: Conclusions

In this study I showed that some fitness related life history traits of *C. elegans*, such as total reproductive output and maturation rate, respond negatively (with one notable exception) both to increasing levels of paraquat and to an increase in culture temperature. I also showed that reproductive output increased at low levels of paraquat in an apparent hormetic effect. In contrast, while lifespan was modified by temperature, a finding supported by previous researchers studies (22), lifespan did not vary in response to paraquat treatment level. The immutability of mean lifespan to the levels of oxidative stress used here provides support against an oxidative stress theory of aging. While quantitative tradeoffs on measured traits in this study were not found, the 44% relative fitness reduction and detected ROS levels were low in worms fed live *E. coli* in high paraquat treated worms during larval and adult stages suggests a tradeoff in terms of antioxidant control and reproduction exists in this system. To untangle the effects of food source, temperature, and oxidative stress on life history traits, I am currently performing a life history assay with live *E. coli* at 20 °C as was previously completed at 25 °C. Additionally, I am in the process of measuring ROS levels in larval worms at 20 °C so as to compare with the same measurements taken at 25 °C. I hope to assess total antioxidant capacity by extending an oxygen radical absorbance capacity assay to this model system which should further elucidate the costs to reproduction in terms of antioxidant capacity in the near future.

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