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A Cost of Reproduction in *Drosophila melanogaster*: Stress Susceptibility

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Abstract

Little is known about physiological mechanisms that underlie the cost of reproduction. We tested the hypothesis that stress susceptibility is a cost of reproduction. In one test of our hypothesis, *Drosophila melanogaster* females were exposed to a juvenile hormone analog (methoprene) to stimulate egg production followed by stress assays. A sterile stock of *D. melanogaster* was employed as a control for reproduction. Exposure of fertile females to methoprene resulted in an increase in female reproduction and increased susceptibility to oxidative stress and starvation (compared to solvent controls). Sterile females did not exhibit a decrease in stress resistance. Mating also stimulated egg production. As a second test of our hypothesis, mated females were compared to virgin females. Mated fertile females were relatively susceptible to oxidative stress, but this relationship was not evident when mated and virgin sterile females were compared. The results of the present study support the hypothesis that stress susceptibility is a cost of reproduction.

Keywords: cost of reproduction, *Drosophila melanogaster*, juvenile hormone, mating, oxidation, starvation, stress

The cost of reproduction is a relationship of central importance in life-history theory (Williams 1966). Demographically, the cost of present reproduction is paid in terms of reduced future reproduction and decreased longevity (Bell and Koufopanou 1986; Stearns 1991). The cost of reproduction has been documented in numerous studies on plants and animals

(reviewed in Roff 1992) in terms of phenotypic correlations, experimental manipulations, genetic correlations, and correlated responses to selection (reviewed in Reznick 1985). However, relatively little is known about the mechanistic underpinnings of this fundamental relationship. Stearns (1991) argued it is important to investigate life-history trade-offs from the viewpoint of mechanisms. It is essential to delineate mechanisms underlying trade-offs to understand how life histories evolve. Life-history trade-offs are thought to evolve around constraints such as limiting commodities (energy, space) or structural components, which can optimize one function but not another simultaneously.

Partridge (1989) identified *Drosophila melanogaster* costs of reproduction associated with egg production and with mating. The cost of egg production (Partridge et al. 1987; Partridge 1989) results in a time lag impact on age-specific mortality (Sgro and Partridge 1999). In addition, exposure of females to males without mating reduces the life span of females (Partridge and Fowler 1990). Mating has a major deleterious effect on female longevity resulting from the transfer of male accessory gland secretion at the time of mating but not from the transfer of sperm (Chapman et al. 1993, 1995). If the female response to the male accessory gland sex peptide is blocked, then the mating cost of reproduction increases (Chapman et al. 1996).

Lines of *D. melanogaster* selected for increased longevity and late-age reproduction have been used to investigate the cost of reproduction. One such set of lines (Rose 1984) showed a correlated increase in starvation resistance and desiccation resistance (Service et al. 1985). In these lines, an underlying physiological basis of starvation and desiccation resistance is increased lipid and glycogen stores (Service 1987; Graves et al. 1992; Djawdan et al. 1996). The accumulation of energy storage compounds in this set of lines suggests that there is a metabolic basis underlying the tradeoff between fecundity and longevity (Djawdan et al. 1996, 1998). Djawdan et al. (1996) and Simmons and Bradley (1997) conducted detailed studies on the energetics of the selected and control lines, which indicate that the long-lived lines store relatively more energy in the body and the highly fecund control line females allocate relatively more energy to reproduction. However, the differences in storage and apparent differences in energy allocation are not readily interpretable in terms of an energy budget because it is not clear how much food is consumed (Bradley and Rose 1998; Rose and Bradley 1998). Chippindale et al. (1993) used these selected and control lines to investigate the effect of dietary manipulation on longevity and starvation resistance. Notably, in relationship to the present study, a short-term stimulation of reproduction by a live yeast diet was associated with a marked loss of starvation resistance.

The value of experimental manipulations for the study of the cost of reproduction is empirically and conceptually well established (Partridge and Sibly 1991). The goal of the present study was to test the effect of experimental manipulation of reproduction on female stress resistance. Exogenous hormone administration and mating were used to stimulate egg production. Treated females were then tested for starvation and oxidative stress resistance. Survival under starvation conditions provides indirect information about lipid reserves in *D. melanogaster* (Service 1987; Bradley and Rose 1998; Djawdan et al. 1996, 1998; Harshman et al. 1999a). Oxidative stress resistance was assayed in the present study because oxidative damage appears to be a cause of aging (Harman 1956; Wallace 1992; Dudas and Arking 1995; Martin et al. 1996; Johnson et al. 1999).

The hypothesis motivating the present study is that there is a trade-off between reproduction and stress resistance. In the present study, hormonal stimulation of egg production in fertile females resulted in starvation and oxidative stress susceptibility. Mating was associated with susceptibility of fertile females to oxidative stress. The results suggest that stress susceptibility is a cost of reproduction in *D. melanogaster*.

Materials and Methods

Stocks and Fly Cultures

Cultures of the Oregon R (OR) and Canton S (CS) stocks and the Orb mutation were maintained at 21°C. OR and CS are commonly used stocks of *D. melanogaster* that have been maintained in the laboratory for decades. Mutations in the *orb* gene (Lantz et al. 1992) are characterized by female sterility resulting from a failure of oocyte maturation at a previtellogenic oocyte stage. The sterile Orb females serve as a control because they can be exposed to agents that stimulate egg production, but they do not produce eggs. Three replicate hormone manipulation experiments were conducted with OR and Orb females, and two hormone manipulation experiments were conducted using CS females. For a comparison of mated and virgin flies, two experiments were conducted with OR females and two experiments were conducted with Orb females.

All stocks were reared on a standard *Drosophila* diet composed of 6.4 g agar, 50 ml molasses, 86 g cornmeal, and 71 g Torula yeast per liter of water. The antifungal agents in the medium were 22 ml of propionic acid and 5.2 g of methyl paraben added to a batch of diet (described above). All three stocks (OR, CS, Orb) were propagated by holding approximately 15 females, with males, in vials on the *Drosophila* diet for 2 days of egg laying at 21°C. Prior to the experiments, males and females were collected within 8 h after eclosion and held at a density of 150 flies per bottle at 21°C for 48 h on the *Drosophila* diet. Males and females were held together for this period, or females were held alone if virgin females were to be tested. Thereafter, the females were used for the treatments that stimulated reproduction and subsequent stress tests.

Hormonal Stimulation of Egg Production by Mated Females

In the context of the present study, the use of hormone administration is a novel approach to study the cost of reproduction. Juvenile hormone and juvenile hormone analogs stimulate yolk protein production (Bownes et al. 1993; Kelly 1994). For all hormone administration experiments, two days after eclosion one set of females was treated with 100 ng of S-methoprene, the biologically active form of the compound, in 0.5 ml of acetone applied to the side of the abdomen. Methoprene was administered to direct contact to the cuticle of each female and the same volume of solvent was applied to solvent-control females. Following external administration, this dose of methoprene is biologically active in terms of stimulating vitellogenesis (Wilson 1983).

To test for toxicity, methoprene was administered to OR females (five replicate vials with 10 females a piece for each doses) using three doses of the juvenile hormone analog (100 ng, 500 ng, 1000 ng) in acetone with acetone alone as a solvent control. These females were the same age and were handled in the same manner as the flies used for the stress

assays. After three days on the standard food, mortality was tabulated. Three days after exposure solvent alone, 8% of the females had died compared to 6% mortality after exposure to 100 ng of methoprene in the solvent. Substantially higher levels of methoprene were employed in some of the toxicity tests treatments (500 ng, 1000 ng) than was used in the experiments (100 ng). The level of female mortality was 6% after exposure to 500 ng of methoprene and 8% after exposure to 1000 ng of methoprene. The incidence of mortality after administration of 100 ng of methoprene to the females used in the experiments was low and approximately the same for each type of female (OR, CS, Orb).

After administration of the hormone analog or solvent alone as a control, experimental females were held on the *Drosophila* food for three days at 21°C at a density of 10 females per vial. These females were transferred to fresh food vials each day. The number of eggs laid by each set of 10 females was counted for each of three days after exposure to the hormone analog or solvent alone. Three days after hormone treatment or exposure to solvent alone, females were tested for resistance to starvation or oxidative stress. In experiment I with OR, some of the females were used to determine the number of vitellogenic oocytes present in females. In experiments II and III with OR, and experiments I and II with CS, the number of eggs were counted for the three days before the stress assays. Orb females were subjected to the same regime of hormone and control administration of solvent.

Virgin Females Compared to Mated Females without Hormone Administration

To allow mating to occur, virgin OR females were held with virgin OR males for two days. Virgin OR females were also held without males for the same period of time. Similarly, virgin homozygous Orb females were held with virgin fertile males from the Orb stock for two days and virgin Orb females were held without males for the same period of time. Females from the CS stock were not used in these experiments. Males were removed from the mixed-sex bottles, whereupon mated and virgin females were transferred to food vials. Each day 10 females (mated or unmated) were transferred to a fresh vial and the eggs produced during the previous day were counted. To assess whether Orb females were mating, vials containing one virgin female and one virgin male apiece were observed for 3 h. During this 3-h observation period, 57% (53/93) of the females were observed to mate and thus we assume that a high proportion of the Orb females used for stress assays were mated after being held with males for two days. Whether virgin or mated, the homozygous Orb females did not produce eggs.

Starvation and Oxidative Stress Assays

Starvation resistance was tested using 10 females in each vial without food at 21°C. Cotton wads saturated with water were used to contain females in vials for the starvation resistance assay. Oxidative stress resistance was tested by holding vials with 10 females in a vial at 21°C with eight filter paper disks soaked with 500 μ l of 5% sucrose solution and 30 mM methyl viologen (paraquat), a compound that generates oxygen radicals. Foam plugs were used to contain females in the vials used for the oxidative stress assay. For both stress tests, mortality was recorded at 6-h intervals until all flies were dead. For both starvation

and oxidative stress assays, five replicate vials with 10 females in each vial were used for each experiment.

Statistical Analysis

The slope of mortality under oxidative or starvation stress conditions was analyzed by regression of log mortality on time. A range of models (distributions) was evaluated in relationship to the pattern of mortality in the present study. The data relatively closely conformed to a gamma distribution. In general, the gamma distribution is useful for analysis of lifetime data when the data are uncensored (Lawless 1982). The data was not normally distributed and thus the generalized linear models (GENMOD) procedure of SAS (SAS Institute, Cary, NC) was used for statistical analysis. As part of GENMOD, generalized estimating equations (GEE) were used to estimate parameters for statistical tests. For any one analysis, on one type of female and one type of stress, a chi-squared test of the homogeneity of the slopes was performed. In addition to the analysis of the slopes of mortality, the time to 50% mortality (LT50) was evaluated by analysis of variance. The time to 50% mortality was calculated as the mean time until half of the flies were dead.

For the LT50 and the slope of mortality, each experiment was analyzed separately, and an overall statistical analysis was conducted that included the replicate experiments for each type of stress and type of female. In no case (slope or LT50) was there a statistically significant experiment-by-treatment interaction, indicating that the treatment effects were consistent for each combination of type of female and type of stress. Consequently, only the results of the overall analyses are presented.

Results

OR, CS, and Orb females were exposed to methoprene or acetone alone and then held on the *Drosophila* diet for three days before the stress tests. Table 1 shows the mean three-day number of eggs or vitellogenic oocytes per female for hormone-treated and control-fertile females. Hormone administration stimulated egg production or the number of vitellogenic oocytes present in females. The vials with Orb females were monitored in all experiments. No eggs were produced by the homozygous Orb females.

Table 1. Egg or vitellogenic oocyte production after application of 100 μ g methoprene or solvent alone as a control in each experiment (Exp.) using fertile females. The values in the table are the three-day mean number of vitellogenic oocytes per female \pm SE (Exp. I Oregon R) or the daily mean total number of eggs produced per female (all other experiments). Vitellogenic oocytes were counted from dissected five-day-old females. For all other experiments, flies were transferred daily to fresh food vials after hormone treatment and the eggs counted for each of three days.

	Oregon R			Canton S	
	Exp. I	Exp. II	Exp. III	Exp. I	Exp. II
Solvent control	26.0 \pm 2.6	7.1 \pm 1.0	29.5 \pm 1.9	15.4 \pm 1.4	22.7 \pm 2.0
Methoprene treated	41.8 \pm 2.7	15.9 \pm 1.3	36.5 \pm 1.1	16.8 \pm 1.1	33.8 \pm 3.8

Figure 1 presents the results of the starvation and oxidative stress tests using OR females in the form of mortality curves for each hormone treatment and solvent control. Each point on the mortality curve is the mean percent cumulative mortality for replicate assay vials (\pm SE). Figure 2 presents the hormone-administration experiments using CS females, and figure 3 presents the hormone-administration experiments for Orb females. The results of the overall statistical analysis of each set of replicate experiments are summarized in table 2. Under stress conditions, CS and OR females die more rapidly after methoprene treatment than they do after treatment with solvent alone. In contrast, the sterile Orb females do not show such an effect.

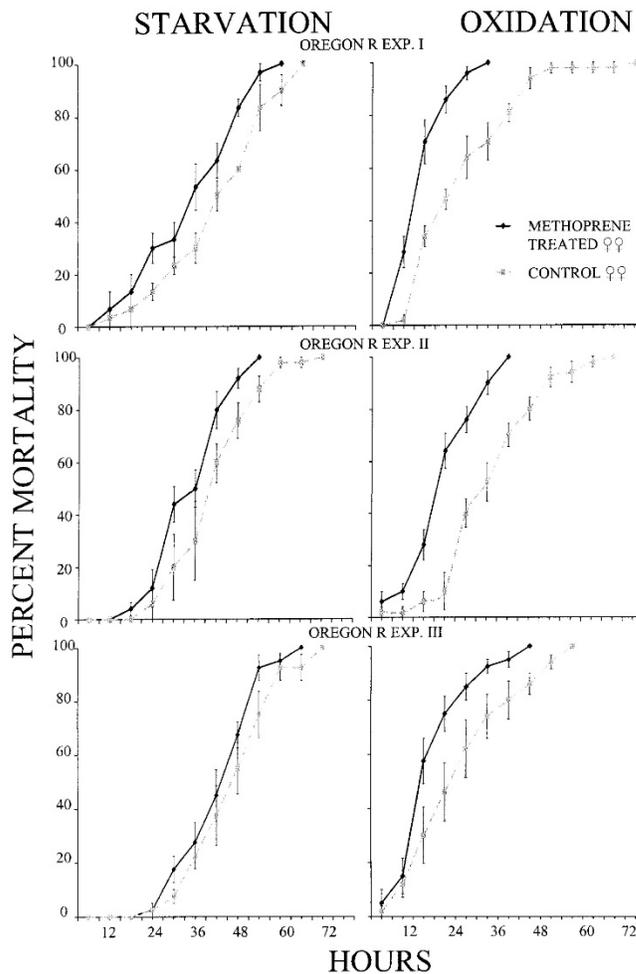


Figure 1. Mortality curves for Oregon R females subjected to starvation and oxidation stresses. Each point on the mortality curve is the mean percent mortality \pm SE of replicate vials. Females were allowed to mate for two days and then treated with methoprene or solvent alone as a control. After treatment, females were kept on standard *Drosophila* medium for three days until assayed. Methoprene stimulated reproduction (table 1) and was associated with relatively steep mortality curves under starvation or oxidative stress.

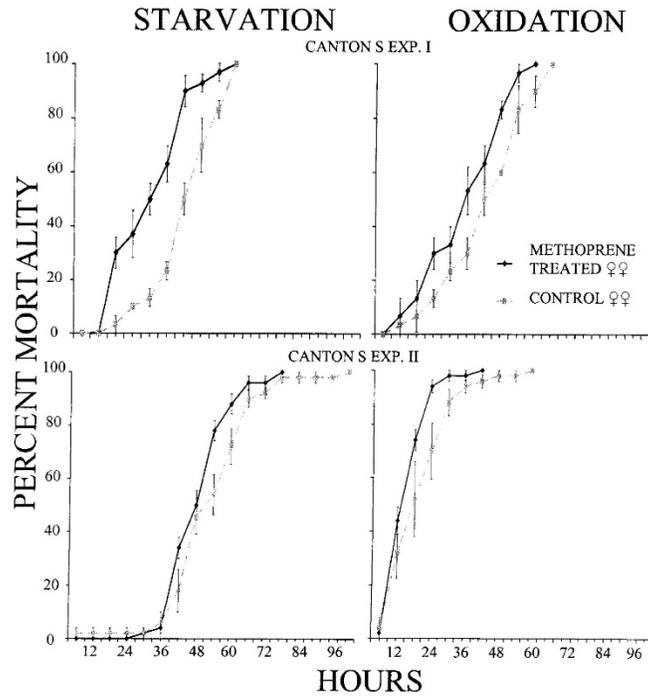


Figure 2. Mortality curves for Canton S females subjected to starvation and oxidative stress. Each point on the mortality curve is the mean percent mortality \pm SE of replicate vials. Females were allowed to mate for two days and then treated with methoprene or solvent alone as a control. After treatment, females were kept on standard *Drosophila* food for three days until assayed. Methoprene treatment stimulated reproduction (table 1) and was associated with relatively steep mortality curves under starvation or oxidative stress.

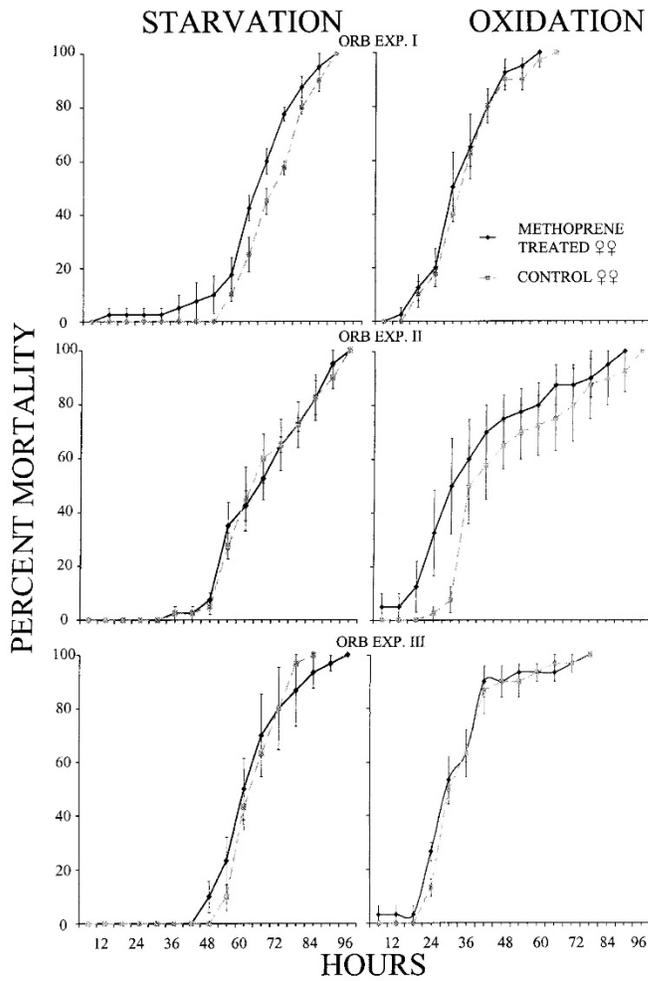


Figure 3. Mortality curves for sterile mutant females subjected to starvation and oxidative stress. The *Orb* mutation is a recessive sterile that results in termination of oocyte maturation at a previtellogenic oocyte stage. Each point on the mortality curve is the mean percent mortality \pm SE. Females were allowed to mate for two days and then treated with methoprene or solvent alone (as a control). After treatment, females were kept on standard *Drosophila* medium for three days until assayed. Methoprene treatment was not associated with stress susceptibility of *Orb* females.

Table 2. *P*-values from the analysis of stress assay data. Female *Drosophila melanogaster* that were treated with a juvenile hormone analog are compared to females treated only with solvent as a control. Oregon R and Canton S females are fertile, homozygous Orb females are sterile and do not produce vitellogenic oocytes or eggs. Three replicate experiments were conducted using Oregon R and Orb females, and two replicate experiments were conducted with Canton S females. The probabilities in the table are from the overall analysis of the replicate experiments for each of the three types of females. The time to 50% mortality (LT50) was analyzed by ANOVA. The slope of mortality was analyzed by regression of log mortality on age.

	Mortality LT50			Mortality slope		
	Oregon R	Canton S	Orb	Oregon R	Canton S	Orb
Oxidative stress	0.0390	0.1248	0.2742	< 0.0001	0.0008	0.1211
Starvation	0.1707	0.2976	0.8699	0.0002	< 0.0001	0.4733

Table 3 shows the daily mean number of eggs (three days total) per female for mated and virgin OR females. Mating stimulated egg production by fertile females.

Table 3. Egg production by Oregon R females prior to stress assays. Females were virgin or were mated (previously held with males). Egg counts were tabulated from replicate vials over three days. The table presents the daily mean \pm SE number of eggs per female.

	Oregon R	
	Experiment I	Experiment II
Virgin females	2.1 \pm 0.7	2.8 \pm 1.1
Mated females	16.6 \pm 0.7	28.9 \pm 2.6

Figure 4 presents the results of the starvation and oxidative stress assays on mated and virgin OR females in the form of mortality curves. Figure 5 presents the results of the starvation and oxidative stress assays using sterile Orb females. Each point on the curve is the mean \pm SE of the replicate vials used for the stress assays. Virgin females were tested at the same time as mated females in each experiment. The probability values from the overall statistical analysis of replicate experiments are presented in table 4. For both fertile and sterile females under starvation conditions, the mortality curves are similar for virgin females and mated females. Under oxidative stress conditions, mated fertile females exhibited relatively high mortality rates compared to virgin females (table 4). Under oxidative stress conditions, there were no statistically significant differences in the slopes of the mortality, or LT50s, for mated Orb females compared to virgin Orb females (table 4).

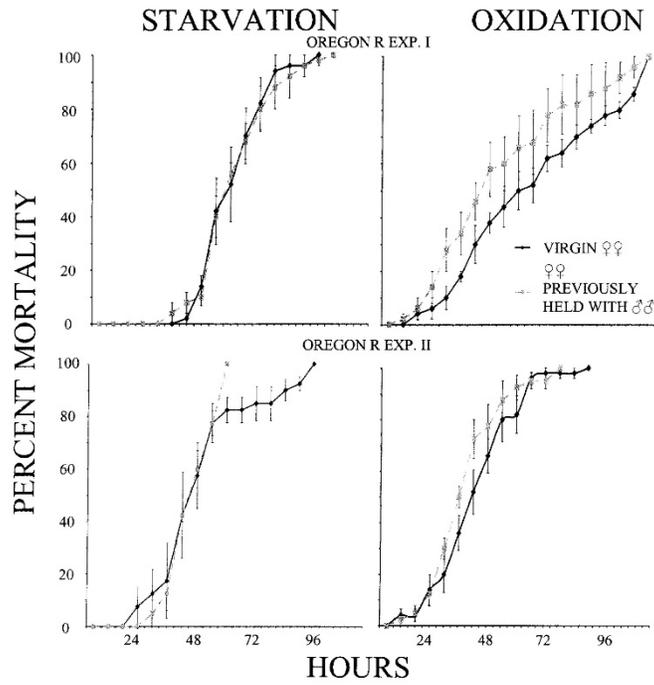


Figure 4. Mortality of fertile Oregon R females without food present or in the presence of methyl viologen as a source of superoxide radicals. Females were either virgins or were mated. Mating, or exposure to males, was associated with oxidative stress susceptibility.

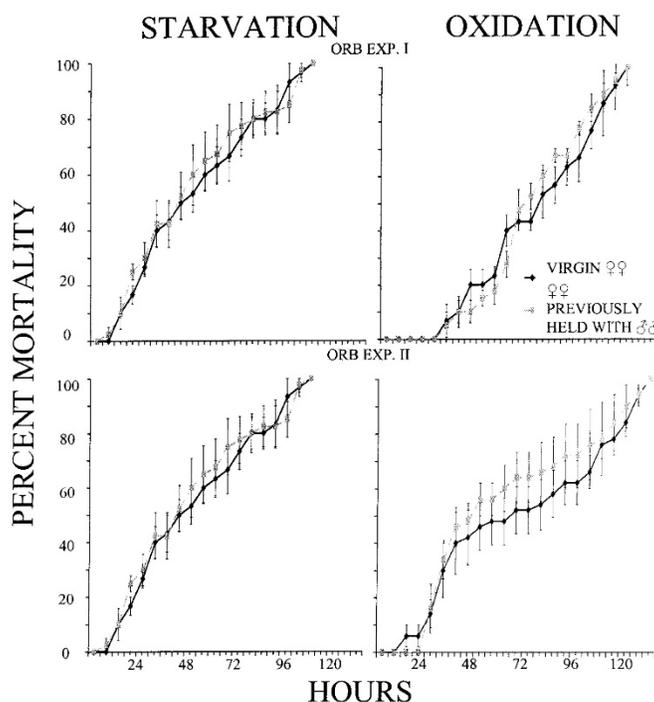


Figure 5. Mortality of sterile females without food present (starvation) or in the presence of methyl viologen as a source of superoxide radicals. Orb is a female sterile mutation that is characterized by termination of oocyte maturation at a previtellogenic stage. Females were either virgins or were mated. Mating, or exposure to males, was not associated with oxidative stress susceptibility of these sterile females.

Table 4. *P*-values from stress assays using female *Drosophila melanogaster* that were mated or virgin. ANOVA was used to analyze the time to 50% mortality (LT50). Regression of log mortality on time was used to analyze the mortality slopes. The *P*-values were derived from the overall analysis of replicate experiments.

Oregon R (fertile females)				Orb (sterile females)			
Oxidation		Starvation		Oxidation		Starvation	
LT50	slope	LT50	slope	LT50	slope	LT50	slope
0.0765	0.0264	0.5980	0.4787	0.1869	0.3003	0.5929	0.7828

Discussion

Oxidative stress susceptibility was a consistent cost of reproduction in the present study. Mating and application of a juvenile hormone analog (methoprene) stimulated egg production, and these treatments were associated with relatively decreased oxidative stress resistance. Stress susceptibility was not observed when methoprene or mating was administered to sterile females, suggesting that oxidative stress susceptibility is linked to egg

production. Mated fertile females were not relatively susceptible to starvation when compared to virgin females, but administration of methoprene to mated fertile females relatively decreased their resistance to starvation. Perhaps extreme depletion of endogenous energy compound stores results from the combined effect of mating and hormone administration. The decrease in starvation resistance observed by Chippidale et al. (1993) after exposure of females to a diet rich in live yeast might arise from depletion of endogenous energy compound stores when egg production by mated fertile females is stimulated.

In terms of the life span of *D. melanogaster* females, exposure to males and egg production incurs a cost of reproduction (Partridge et al. 1987). A longevity cost of female reproduction in *D. melanogaster* is associated with mating (Fowler and Partridge 1989) and is largely produced by male accessory gland products (Chapman et al. 1995). In the present study, OR females did not produce eggs, and they did not exhibit increased oxidative stress susceptibility as a function of previous exposure to males and/or mating. Thus, oxidative stress susceptibility is apparently associated with egg production. In future studies, it will be interesting to determine if and how the oxidative stress susceptibility cost of reproduction is related to the longevity cost of reproduction.

In the field of aging research, several factors predict longevity. The first factor is stress resistance. Based on mutation analysis, selection experiments, and studies with transgenic organisms, stress resistance tends to be correlated with greater longevity (Rose 1984; Service et al. 1985; Rose et al. 1992; Lithgow et al. 1995; Tatar et al. 1997; Johnson et al. 1999). In particular, oxidative stress might play a general role in the aging process, and resistance to oxidative stress might be a longevity factor (Orr and Sohal 1992; Lithgow and Kirkwood 1996; Sun and Tower 1999). However, it is increasingly clear that stress resistance and increased longevity are not necessarily associated (Harshman et al. 1999b; Clancy et al. 2001). The second factor that predicts longevity is reproduction, which is correlated with reduced longevity. A connection between these factors is suggested by the disposable soma theory of aging (Kirkwood 1977; Kirkwood and Rose 1991; Kirkwood and Franceschi 1992). The theory argues that when endogenous resources are allocated to reproductive function (e.g., gametogenesis), they are not available for somatic maintenance functions, such as stress resistance. In the present study, application of methoprene to fertile females stimulated egg production. After hormone administration, increased egg production was associated with decreased starvation resistance and decreased oxidative stress resistance. This result could be interpreted in terms of the disposable soma theory. Specifically, it is plausible that increased egg production resulted in a shift of lipid reserves from the body for the purpose of reproduction. In fact, the relative measure of starvation resistance could be a proxy measurement for decreased energy available for somatic maintenance and protection from oxidative stress in the present study. However, mating was associated with a large increase in egg production and decreased oxidative stress resistance in OR females without a concomitant decrease in starvation resistance (fig. 4).

What else could account for the stress susceptibility cost of reproduction observed in our study? A signal from the reproductive system might alter the expression of genes responsible for somatic maintenance and repair, or reproduction might directly cause somatic damage (Sgro and Partridge 1999), which could result in stress susceptibility. It is potentially relevant that ablation of cells that give rise to the reproductive system of the

worm *Caenorhabditis elegans* obviates the life-span extension of an insulin-signaling mutation (Hsin and Kenyon 1999). A signal from the reproductive system to the soma might contribute to stress susceptibility. In present study, increased egg production is associated with stress susceptibility, but it is not clear that this cost of reproduction is caused by the energetic cost of egg production. In general, the present study provides support for the hypothesis that stress susceptibility is a cost of reproduction.

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