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INFLUENCE OF MATERNAL AGE ON THE EFFECTS OF SELENO-L-METHIONINE IN THE MODEL ORGANISM *DAPHNIA PULEX* UNDER STANDARD AND HEAT STRESS CONDITIONS

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Abstract

Selenium deficiency and toxicity increase the risk of adverse developmental and reproductive outcomes; however, few multi-stressor studies have evaluated the influence of maternal age on organic selenium dose-response and additional stressors over the life course. While multi-stressor research in mammalian models is time-consuming and expensive, use of alternative models can efficiently produce screening data for prioritizing research in mammalian systems. As a well-known eco-toxicological model, *Daphnia pulex*, may offer advantages in screening for impacts of multi-stressor exposures. We evaluated the influence of maternal age on the effects of seleno-methionine (SeMet) for lifespan, reproduction, and heat-stress resistance in *D. pulex*. Our results show effects of SeMet-treatment and maternal age, where the highest SeMet-treatment had reduced lifespan and absence of reproduction, and where *Daphnia* from late life broods had increased resistance to heat-induced stress. Further analysis suggests an additional interactive effect between maternal age and SeMet treatment on time to first reproduction.

Keywords

metals; maternal effect; selenium; daphnia; hyperthermia; multi-stressor

1. Introduction

Multi-stressor analyses are necessary to understand the interactive effects of the stressors that organisms experience in their environments¹. However, the complex nature of these

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research questions in mammalian models can result in extensive, time consuming, and costly experiments. Thus, alternative non-vertebrate animal models may offer an efficient and cost-effective method for screening multi-stressor hypotheses over the lifespan. The utility of the freshwater crustacean, *Daphnia pulex*, is currently being evaluated as a screening model for the chronic effects of multi-stressor exposures²⁻⁴. A well-known eco-toxicological model system, *D. pulex* has been used since the turn of the 20th century to study the mechanistic nature of environmental factors such as uptake of trace metals⁵. It is a model organism for biomedical research with advantageous traits for chronic toxicity testing including parthenogenic (clonal) reproduction, short lifespan (60 days), well-characterized ecology and response to environmental stimuli, tight coupling of somatic growth and reproductive output, ease of laboratory culture maintenance, measureable span of reproduction cycle, release of live offspring every 24 to 48 hours, transparency throughout adulthood, and recently developed genomics tools⁵⁻⁸. *Daphnia* is being used to study human pathogen virulence, aging, oxidative stress responses, and xenobiotic detoxification⁹. Recently, Mawell et al. identified greater similarities between human and daphnia disease gene clusters than those found in other validated invertebrate model organisms for biomedical research, *C. elegans* and *D. melanogaster*¹⁰.

Maternal aging may play a key role in the life history success of offspring. Particularly, maternal effects may provide a mechanism for adaptive transgenerational phenotypic plasticity^{11,12}. For instance, advanced maternal age at conception is suspected of leading to a variety of health complications¹³⁻¹⁷ and shorter lifespans¹⁸⁻²⁰ in progeny. Offspring fecundity is also shown to decrease with maternal age due to decreasing physiological health of the mother's reproductive system and damage incurred to her gametes²¹. Parthenogenic (clonal) model organisms, such as *Daphnia pulex*, are particularly advantageous for elucidating maternal age effects due to limited genetic variation across offspring²². Previous studies have demonstrated these maternal effects in *D. pulex*. For example, maternal food environments are shown to affect reproduction in offspring²³ and maternal thermal environments are shown to affect offspring resistance to *Microcystis*¹².

Although data in mammalian models currently exists for selenium (Se) deficiency²⁴⁻²⁶ and toxicity²⁷⁻²⁹, little is known about the interactions between Se toxicity and other stressor exposures. Animal studies across various taxa show that chronic concentrations of elevated Se result in developmental abnormalities^{30,31}, reduced reproduction^{30,32-34}, and generation of reactive oxygen species (ROS)³⁵. Particularly, the Se form Seleno-methionine (SeMet) is speculated to have increased risk for chronic toxicity³⁶ due to incorporation into proteins and low levels of excretion³⁰. At high SeMet concentrations, Se body pools are known to increase, but specific mechanisms underlying Se toxicity are not well characterized³⁷. A recent study in zebrafish suggests oxidative stress and methylation pathways may play a role in SeMet-induced toxicity³⁸.

At low supplemental doses, organic Se supplementation increases the immune response and lessens the physiological stress related to hyperthermia^{39,40}, which is commonly associated with increasing the risk of mortality⁴¹ and exacerbating effects of disease^{42,43}. Hyperthermia results in altered protein synthesis and enzymatic expression changes in various organ tissues such as muscles, heart, brain, kidney liver, and lungs⁴⁴. Many proteins,

including stress-induced phosphoprotein 1, suppression of tumorigenicity 13, and Hsp90 beta, exhibit higher expression levels when exposed to elevated environment temperatures⁴⁰. Particular physiological responses to heat exposures include proteolysis regulated decreases in protein degradation and increased creatine kinase enzyme presence. Studies of white sturgeon revealed that SeMet supplementation has the capacity to attenuate this effect of proteolysis, thus, potentially promoting an increase in protein degradation due to heat exposure⁴⁰. SeMet supplementation trials of swine using nontoxic doses also improved the ability to resist heat-induced stress³⁹. In *Daphnia*, utilization of thermal stress has been used to exacerbate toxic response. For instance, *Daphnia* dually exposed to cadmium and elevated temperature exhibited increased sensitivity⁴⁵. Recorded responses of *Daphnia* to elevated temperatures include changes in swimming behavior, immobilization, and death⁴⁶. The effect of SeMet on thermal stress has not yet been evaluated in *Daphnia*.

The present study builds on this previous literature by utilizing SeMet toxicity as well as the potential for SeMet to mitigate effects by heat-induced stress to elucidate the influence of maternal age in a multi-stressor analysis. The purpose of this study is to investigate the influence of maternal age on the effects of organic selenium (SeMet) in *D. pulex* on lifespan, reproduction, and resistance to heat-induced stress. We hypothesized that *D. pulex* obtained from older maternal populations will express exacerbated effects of SeMet toxicity and decreased efficiency of SeMet protection under hyperthermia conditions. We evaluated the interactions between chronic SeMet exposure, maternal age, and resistance to acute heat stress in *Daphnia pulex*.

2. Materials and Methods

2.1 Model Organism and Culture Media

The *Daphnia pulex* cultures were established from a clonal population obtained from Indiana University⁶. Populations of animals were maintained under previously established laboratory culture protocol conditions^{2-4,6}. All *D. pulex* cultures were maintained in COMBO media at a density of 20 animals per 1 L media. COMBO media and Animate stock solutions were prepared as described by Kilham, Kreeger, Lynn, Goulden, & Herrera, 1998. To prepare COMBO media, Calcium Chloride (CaCl₂, Acros Organics, 99% purity, CAS:7447407, lot:A0294654), Magnesium Sulfate (MgSO₄, Fisher Scientific, 100.6% purity, CAS:10034998, lot:150090), Sodium Bicarbonate (NaHCO₃, Acros Organics, 99% purity, CAS:7647156, lot:A0289787), Sodium Metasilicate (Na₂SiO₃, Acros Organics, 47.5% total solids, CAS:13517243, lot:A0292413), Potassium Chloride (KCl, Fisher Scientific, 99% purity, CAS:7447407, lot:105143), and Boric Acid (H₃BO₃, Fisher Scientific, 100.1% purity, CAS:10043353, lot:106357) stock solutions were added (1ml/L) to MilliQ ultrapure deionized water and allowed to aerate for at least 12 hours prior to use. Immediately prior to media changes, 2.18 µg/L Sodium Selenite (Na₂SeO₃, Acros Organics, 44–46% Se, CAS:10102188, lot:A0270903), 1ml/L Animate stock solution (LiCl, Fisher Scientific, 98.5% purity, CAS:7447418, lot:107322, RbCl, Acros Organics, 99.8% purity, CAS:7791119, lot:A0279017, SrCl₂ 6H₂O, Acros Organics, 99% purity, CAS:194122500, lot:A0262200, NaBr, Acros Organics, 99% purity, CAS:7647156, lot:A0294885, KI, lot:A0273463), and RGcomplete™ microalgae concentrate (Reed Mariculture Inc.,

Campbell, CA 95008), were added to the media batch. For experimental populations, SeMet ($\text{CH}_3\text{SeCH}_2\text{CH}_2\text{CH}(\text{NH}_2)\text{COOH}$, Sigma Aldrich, 98% purity, CAS:3211765, lot:S3132) was substituted for NaSeO_3 as described in section 2.3. Complete media changes were performed every 48-hours unless otherwise stated. *Daphnia* were kept in 1 L beakers (20 daphnids per 1 L) and housed in a Percival I-36NL environmental chamber to regulate temperature ($22.5^\circ\text{C} \pm 0.05^\circ\text{C}$) and light conditions (12-hour light: 12-hour dark cycle).

2.2 Dose-Finding Study

D. pulex were cultured under standard laboratory maintenance protocol with the exception of substituting SeMet for Na_2SeO_3 during regular media changes. A total of five SeMet treatment concentrations were analyzed: 0- μg SeMet/L, 1- μg SeMet/L, 2- μg SeMet/L, 4- μg SeMet/L, and 8- μg SeMet/L. The highest treatment (8- μg SeMet/L) is the concentration of SeMet at which 50% of the population were dead (LD 50) in adult *Daphnia magna* after a 48-hour exposure⁴⁸. A 50% dilution was applied to obtain the remaining treatments. In the control group (0- μg SeMet/L) no additional Se was added. Each SeMet treatment was assessed using a population of $n=20$ individuals, that were followed as populations throughout their lifespan. Mortality and presence of offspring were recorded throughout the lifespan of each population.

2.3 Seleno-methionine Exposure

Three SeMet doses (0- μg SeMet/L, 1- μg SeMet/L, and 4- μg SeMet/L) were selected based on the ability to monitor effects over the lifespan as well as the presence of a trending deviation in lifespan and reproduction between doses, see section 3.1. The highest treatment (8- μg SeMet/L) was excluded due to high rates of early life mortality.

The SeMet stock solution was prepared prior to the initiation of the experiment by dissolving 10 mg seleno-L-methionine (Fisher Scientific) into 1 L deionized MilliQ ultrapure water. This stock solution was added at specific concentrations, respective to the treatment, to each batch of prepared media in place of inorganic Se (Na_2SeO_3) typically added, see section 2.1. The base media (COMBO, animate, and algae) was prepared as a single batch, aliquoted into the three SeMet treatment groups, and the designated SeMet stock solution concentration was added. The media was then aliquoted into the appropriate exposure containers (glass beakers or polypropylene conical tubes) for group or individual exposures. Media volumes were adjusted based on the population density during each media change (50 ml media per *daphnid*). For each experiment, *D. pulex* neonates began exposure to 0- μg SeMet/L, 1- μg SeMet/L, or the 4- μg SeMet/L within 24 hours of age. Fresh media containing appropriate concentrations of SeMet was supplied every 48-hours during media changes until the conclusion of the experiment.

2.4 Selenium Content Analysis

Daphnia ($n=108$)[3 SeMet treatments $\times$ (3 replicates $\times$ 12 individuals per replicate)] were cultured in standard maintenance media, 0- μg SeMet/L, 1- μg SeMet/L, or 4- μg SeMet/L treatments until 11 to 12 days of age. Each animal was then rinsed 3 times with DI water and submitted to the Virginia-Maryland College of Veterinary Medicine Toxicology Lab for quantification of Se content by atomic absorption spectrometry. Samples of COMBO media

and COMBO + RG Complete were also submitted for analysis. Briefly, all glassware used was acid-washed and samples were transferred to a glass beaker by adding a mixture of perchloric acid/nitric acid (about 10 ml). The mixture was heated at 250 °C until the volume reached ~ 2 ml. Samples were transferred to 20 ml glass tubes in a final volume of 7 ml of 20% hydrochloric acid. After 1 hour, samples were analyzed by atomic absorption spectrometry with a vapor generator accessory (Wavelength: 196.0 nm).

2.5 Maternal Age

Daphnia pulex maternal populations were established from the third brood of our laboratory maintenance *D. pulex* culture (maternal age ~ 12 days). Neonates (~ 24 hours of age; n=80) were obtained from the maintenance population and randomized into four maternal populations. These maternal populations each consisted of n=20 individuals that were housed in 1 L beakers containing 1,000 ml COMBO media and allowed to age under standard laboratory maintenance conditions. All experimental animals exposed to SeMet were obtained from these maternal populations when the mothers were 8-days of age (early life (2nd–3rd) brood) and when the mothers were 32-days of age (late life (12th–14th) brood).

For the lifespan analysis, the offspring from all four maternal populations were combined into a single pool from which offspring were randomized into three SeMet treatment groups [(1 maternal population × 3 SeMet treatments) × 25 replicates per SeMet treatment]. This was conducted once when the maternal populations produced an early life brood and repeated when the maternal populations produced a late life brood. For the heat induced stress analysis, offspring were tracked by maternal population. Within the maternal population tracks, offspring were randomized into the three SeMet treatments and housed as populations, creating one experimental group for each maternal population. This was repeated for replicate groups to ensure that each SeMet treatment is assessed in 8 populations for each maternal age group; giving a total of n=24 exposure groups per maternal age [(4 maternal populations × 3 SeMet treatments) × 2 replicates]. Diagram presentations of the experimental designs are depicted in Figure 1, Figure 2, Figure 3.

2.7 Lifespan and Reproduction

At each of the two maternal age time points, *D. pulex* neonates (~ 24 hours of age, n=75) were obtained from the maternal populations and randomized into the three SeMet treatment (n=25 per SeMet treatment per maternal age). Each neonate was placed into a separate 50 ml polypropylene conical tube containing 50 ml of media with the designated SeMet concentration. Mortality and reproduction were recorded every 48-hours. The number of combined living and dead offspring produced over the individual's entire lifespan (total reproduction) and the first day offspring were present in the media (time to first reproduction) were recorded for each individual. Individuals that did not survive to reproductive age or did not reproduce were censored from this analysis. Thus, of the 150 neonates combined across all treatments, a total of 98 individuals produced offspring and were included in the time to first reproduction and total reproduction analyses unless otherwise stated. This experiment was performed first for offspring obtained from the early life brood and then repeated for the offspring obtained from the late life brood.

2.8 Heat Induced Stress Analysis

At each of the two maternal age time points, *D. pulex* neonates (24 hours of age, n=360) were randomized into 24 exposure populations tracking by maternal population. Fifteen individuals were maintained per exposure population in 1 L glass beakers containing 750 ml of media with the designated concentration of SeMet. The neonates were allowed to age in their designated treatment group for 15-days, changing the media every 48-hours as previously described. Heat exposure temperature, 35°C, and heat exposure duration, 30 minutes, were determined by conducting preliminary trial heat stress experiments, in which produced 80 percent mortality rates, utilizing *D. pulex* (15 days of age) obtained from our laboratory maintenance populations. This experiment was conducted when the maternal populations produced their early life brood and then repeated for the late life brood.

The heat stresses were conducted in pre-heated (35° C) water baths. Once the water baths reached the designated temperature, *D. pulex* were moved from their maintenance media (at 24° C) and put into 100 ml glass beakers containing 100 ml media (at room temperature) in groups of 10 individuals per beaker. Media was prepared without algae (food) and only contained COMBO, animate, and the appropriate concentration of SeMet. The beakers containing the *D. pulex*, were then placed directly into the water bath and were allowed to reach 35° C (approximately 30 minutes). Once the water within the beakers reached 35° C, the 30-minute heat exposure time began. After removal from the water baths, the beakers containing *D. pulex* were placed back into the environmental chamber and algae was added to the media. The daphnids were allowed to recover for a total time of 24-hours. Mortality and presence of offspring were recorded at 4-hours and 24- hours post heat stress.

2.9 Statistical Analysis

Statistical analysis was conducted using JMP Pro 12.0.1 and IBM SPSS Statistics 24 JMP Pro 12.0.1 computer software. Differences in mortality between SeMet treatment and maternal age groups were determined by constructing a Kaplan-Meier (survival) distribution and testing for significance between groups with the Log rank test. Cox proportional hazards models were used to test for interactions between SeMet exposure and maternal age in lifespan, time to first reproduction, and heat-induced stress mortality. Two-way ANOVAs were constructed to compare the effects and interactions of SeMet treatment and maternal age for measured reproductive parameters total reproduction (lifespan analysis). Differences between SeMet treatment groups or maternal population ages for the reproductive measures were determined using Tukey's honest significant difference (HSD) test. Chi-Squared were used to assess the presence of offspring post heat-induced stress. All statistical results were considered to be significant if $p \leq 0.05$.

3. Results

3.1 Seleno-L-methionine Exposure Dose-Finding Study

This study revealed a significant dose-response effect among SeMet/L treatments where increasing SeMet concentration corresponded with higher mortality rate over the lifespan ($\chi^2(4,100) = 58.92$, $p < 0.0001$; Figure 4). Lifespan was shortest for the 8- μ g SeMet/L treatment (Mdn $\pm$ SE = 11.0 ± 0.85 days) in comparison to all other treatment groups (Mdn

$\pm$ SE = 41.0 ± 8.2 , 39.0 ± 2.9 , 23.0 ± 3.0 , 27.0 ± 1.7 for 0, 1, 2, and 4- μ g SeMet/L respectively). *Daphnia* exposed to the 0 and 1- μ g SeMet/L had longer lifespans than the *daphnia* exposed to 2 and 4- μ g SeMet/L. No differences were observed in survival between the 0 versus the 1- μ g SeMet/L or between the 2 versus the 4- μ g SeMet/L. The *Daphnia* in the 0 versus the 1- μ g SeMet/L groups had mortality rates that intersected during midlife (30–35 days), calculated as 50 percent of the average laboratory maintenance population lifespan (60–70 days; Figure 4). A similar pattern was seen between the *Daphnia* exposed to 4- μ g SeMet/L versus the *Daphnia* exposed to 2- μ g SeMet/L.

3.2 Selenium Content in *Daphnia* and Media

Based on the dose finding study, subsequent analyses focused on 0, 1, and 4- μ g SeMet/L treatments. Evaluation of Se levels in daphnia exposed to each treatment for 12-days show elevated concentrations in 1 and 4- μ g SeMet/L treatment groups (median concentrations 746 ppb and 2383 ppb, respectively) when compared to standard media and 0- μ g SeMet/L treatment groups (Figure 5). Se concentrations measured in COMBO media and COMBO +RG-Complete algal feed were 2 ppb and 11 ppb, respectively.

3.3 Lifespan

A significant dose-response trend is present among SeMet/L treatments (Mdn = 40.0 ± 4.1 , 44.0 ± 3.5 , 31.0 ± 1.8 days for 0, 1, and 4- μ g SeMet/L respectively) where increasing SeMet concentration corresponded with higher mortality rate over the lifespan (X^2 (2, 75) = 45.5, $p < 0.0001$; Figure 6). All treatment groups had similar mortality rates early in life. As they approach mid-life (30 days), the mean lifespan of *D. pulex* in the 4- μ g SeMet treatment starkly declined. However, the difference in mortality rate between 0 and 1- μ g SeMet/L treatments diminishes as the *Daphnia* continued to age (Figure 6). There was no significant effect of maternal population age alone on lifespan (X^2 (5, 75) = 45.5; $p=0.66$; Table 1). The interactive effect of maternal age on SeMet treatment was non-significant (X^2 (5, 75) = 45.5; $p=0.44$; Table 1).

3.4 Reproduction

3.3.1 Total Reproduction—Overall, SeMet treatment presented a significant effect on total reproduction (F (5, 75) = 23.5, $p < 0.0001$; Table 1) where the highest SeMet treatment (4- μ g SeMet/L) resulted in an absence of reproduction. Due to this lack of reproduction, the highest SeMet treatment group was excluded from further analysis on reproductive parameters. An interaction between SeMet treatment groups (0- μ g SeMet/L and 1- μ g SeMet/L) and Maternal Age was not present in total reproduction ($p = 0.41$; Table 1). Significant differences were not found between maternal age populations (Early life brood mean: 256 ± 18 , Late life brood mean: 282 ± 18 ; $p = 0.32$; Table 1) or between the remaining SeMet treatments for the 0- μ g SeMet/L and 1- μ g SeMet/L (mean = $(272 \pm 21$ and 266 ± 14 respectively; $p = 0.80$).

3.3.2 Time to First Reproduction—*Daphnia* exposed to 4- μ g SeMet/L were censored from this analysis due to their lack of reproduction. A significant interaction was present between Maternal Age and SeMet treatments for time to first reproduction (X^2 (3, 98) = 55.6, $p < 0.0001$; Figure 7; Table 1). *Daphnia* obtained from older maternal populations had

delayed time to first reproduction with increased SeMet treatment than younger maternal populations. The pattern is as follows, individuals that were exposed to 0- μ g SeMet/L began reproducing earlier in life if they were obtained from a later life brood (Early life brood mean: 9.2 days, 95% CI [8.2, 10]; Late life brood mean: 8.0 days, 95% CI [7.0, 9.0]); whereas *Daphnia* exposed to 1- μ g SeMet/L began reproducing later if they were obtained from a later life brood (Early life brood mean: 11 days, CI 95% [10, 12]; Late life brood mean: 13 days, CI 95% [12, 14],).

3.4 Heat Induced Stress

3.4.1 Survival—Overall, there was a significant increase in days survived post-heat stress in *Daphnia* obtained from older maternal populations (mean = 1.2 ± 0.2 and 2.8 ± 0.2 days for early life brood and late life brood respectively, $X^2(6, 480) = 20.5$, $p = 0.002$; Table 1) suggesting that offspring obtained from early life brood are less resistant to heat stress. However, there was no significant effect of SeMet treatment (mean = 2.1 ± 0.3 , 1.6 ± 0.2 , and 2.3 ± 0.3 days for 0- μ g SeMet/L, 1- μ g SeMet/L, and 4- μ g SeMet/L treatments respectively, $p = 0.12$; Table 1) or an interaction between SeMet treatment and maternal age ($p = 0.10$; Table 1).

A trending effect on maternal age occurred where individuals from the late-life brood lived longer after the heat stress than the *Daphnia* from an early-life brood across all SeMet treatments. By 4 hours post heat stress, 74% of the individuals obtained from the early-life brood and 38% of the individuals obtained from the late-life brood were dead. However, by 24 hours post heat stress, nearly 100% of the remaining *Daphnia* were dead (98% mortality in *Daphnia* obtained from a late-life brood, 99.6% mortality in *Daphnia* obtained from an early-life brood). An interesting inverse response pattern (not significant) was present in the *Daphnia* exposed to 4- μ g SeMet/L in relation to the other SeMet treatments based on maternal age. In the *Daphnia* obtained from an early-life brood, the individuals exposed to 4- μ g SeMet/L had higher percent survival to the heat-induced stress than the other SeMet treatment groups at 4 hours post heat stress. Whereas, in the *Daphnia* obtained from a later life brood, the individuals exposed to 4- μ g SeMet/L had the lowest percent survival of the SeMet treatment groups at 4 hours post heat stress.

3.4.2 Presence of Reproduction after Heat Stress—Similar to the earlier reproduction analysis, *D. pulex* exposed to the 4- μ g SeMet/L did not reproduce and were censored from further analysis. No offspring were found at 4-hours post heat stress. However, at 24-hours post heat stress (nearly 100% maternal mortality), all late-life brood populations and a large portion of the early-life brood populations contained offspring (100% and 56% of populations containing offspring in the late-life broods and early-life broods respectively). An effect of maternal age on presence of reproduction was observed ($X^2(1, 32)$, $p = 0.003$; Table 1) where offspring presence was higher for *Daphnia* obtained from late-life broods. An effect of SeMet treatment (87% and 68% of populations containing offspring in the 0- μ g SeMet/L and 1- μ g SeMet/L treatments respectively; $X^2(1, 32)$, $p = 0.2$) or an interaction between maternal population age and SeMet treatment were not found ($X^2(3, 32)$, $p = 0.08$; Table 1).

Among the *D. pulex* obtained from an early-life brood, there was an interesting pattern of difference in offspring presence between the SeMet treatment groups. In the early-life broods, 75% of the populations exposed to 0- μ g SeMet/L treatment produced offspring whereas only 38% of the populations exposed to 1- μ g SeMet/L treatment produced offspring. This suggests that in the *Daphnia* obtained from young mothers, the ability to produce viable offspring may decrease with increasing SeMet treatment during a heat-induced stress. However, this pattern was only seen in the *Daphnia* obtained from young maternal populations.

4 Discussion

The presented research uses the model system *Daphnia pulex* to investigate potential multi-stressor interactions between maternal age and response to SeMet treatment. To our knowledge, this is the first study that uses *D. pulex* to investigate this particular interaction over the lifespan. This study is relevant to the development of alternative screening models in toxicity testing and maternal aging research. Increased abundance of Se in our environment from use of Se supplementation, production of Se wastes, use of Se containing fertilizers, and elevated prevalence of later life pregnancies in many human populations make this multi-stressor hypothesis a relevant human concern.

The purpose of this study was to evaluate a multi-stressor hypothesis utilizing heat-induced stress to exacerbate any maternal age and SeMet treatment interactions. In this study, a significant interaction of maternal age and SeMet treatment was seen for time to first reproduction. Under control conditions (0- μ g SeMet/L), offspring obtained from advanced maternal age exhibited earlier initiation of reproduction. Whereas, SeMet treatment produced a trending delay in time to first reproduction in all individuals with more exaggerated effects found in offspring obtained from advanced maternal age. This suggests a difference in SeMet dose-response based on maternal age. At 0- μ g SeMet/L, the *daphnia* from both early and late life broods began reproducing at similar age ranges seen in maintenance populations (6 to 9 days). However, by increasing the SeMet dose, reproduction was delayed. This delayed effect caused by SeMet treatment could be a resulting coping mechanism by which the mother transfers her Se body load to an undeveloped egg and expels it as waste as similarly seen in mammalian systems³². The *daphnia* from the early-life broods were able to efficiently cope with the elevated levels of SeMet. In contrast, the *daphnia* from the late-life brood were not able to efficiently cope with the elevated levels of SeMet and thus had delayed reproduction. Similarly, an interaction between maternal age and ethanol exposure was seen in a study conducted by Vorhees (1988), where mice born from older mothers had increased effects of prenatal ethanol treatment including increased mortality, lower weight gain, altered olfactory orientation, and altered behavior⁴⁹. In contrast, Martínez-Jerónimo et al. (2006) did not detect a significant effect of maternal age on lifespan in response to the toxicity of hexavalent chromium in *Daphnia magna*⁵⁰.

A significant interaction of maternal age and SeMet treatment was not detected in response to heat stress. However, older maternal ages did result in an overall increased resistance to heat induced stress regardless of SeMet treatment. It is possible that the difference in percent mortality post heat stress based on maternal age we observed in this study may be due to a

maternal aging effect specific to maternal size and ability to produce larger offspring. *Daphnia* have the ability to continue to grow throughout their adult life, increasing the size of their brood pouch along with the potential size of their offspring as they age^{50,51}. Larger offspring body sizes from older mothers could potentially increase their ability to maintain homeostasis during a heat stress. This finding is consistent with past studies showed that maternal age was positively associated with heat stress resistance (and offspring body size) in *Drosophila mercatorum*⁵² and *Daphnia similoides*¹². The effect of maternal age should not be ignored in utilization of *Daphnia* as an alternative animal model and should be further explored in future research. Further, we found a lack of a main effect of maternal age on lifespan and total reproduction. This is inconsistent with previous aging studies in *Drosophila melanogaster*²⁰ and humans⁵³ where advanced maternal age resulted in decreased lifespans and decreased reproduction in the offspring.

This study is also the first to our knowledge to quantify the effects of chronic exposure to SeMet in *D. pulex* utilizing lifespan and reproductive measures, which is relevant to the development of alternative whole animal screening models for chronic low-dose exposures. As in mammals, Se is an essential element for *Daphnia* in development and reproduction⁵⁴, and critical points in reproduction are hormonally controlled via interaction with nuclear receptors^{55,56}. Based on this study, low deviations in Se concentrations administered as SeMet over the lifespan may have the ability to induce negative responses on mortality and reproduction that are not detectable under acute exposure conditions. We found that at the highest SeMet treatment tested (4- μ g SeMet/L), overall lifespan significantly decreased and reproduction was inhibited. This was similarly observed in female rats and fish, where excess Se supplementation led to accumulation of Se in organs and tissues of reproductively active females resulting in complete reproductive failures³⁰. We did not detect deficiency effects in animals exposed to 0- μ g SeMet/L, possibly as a result of sufficient levels of Se present in the algae or sufficient maternal transfer of SeMet into the offspring during development.

It should be noted that the animals in our dose-finding study did deviate in response to SeMet in comparison to a previous study in *Daphnia magna*. According to Ingersoll et al. (1990), the 48-hour LD₅₀ for SeMet is 8- μ g SeMet/L in *Daphnia magna*⁴⁸. However, in our study, we did not observe high rates of mortality in the 8- μ g SeMet/L (or lower SeMet treatments) within the first 48-hours of exposure study. Rather, we saw an increase in mortality over the lifespan and a stark inhibition of reproduction with elevated SeMet treatment. We believe that differences in the investigated parameters in the study of Ingersoll et al. (1990), such as *Daphnia* species, age of animals, diet, and length of exposure, may account for the discordance with our study.

For this experiment, *Daphnia* were exposed to SeMet via introduction of a laboratory prepared aqueous stock solution to the media; however, exposure via dietary consumption would be more consistent with human studies. For the purposes of our study in *Daphnia pulex*, this was not considered to affect the overall outcome of the study. A previous study in *Daphnia magna* suggested that dietary exposure via algae versus aqueous exposure did not significantly affect SeMet bioaccumulation⁵⁷. Decreasing the time between heat stress observations and increasing the sample size would also have improved the statistical power

of the experiment. An additional limitation was the use of maternal populations rather than individual mothers to produce experimental offspring. Even though parthenogenic reproduction produces clonal replicates of the mother ⁵, it is still possible that variation in maternal transfer of Se to offspring could be present between the mothers of each population. Obtaining offspring from individual mothers rather than maternal populations may decrease variation (e.g. due to developmental stage differences in heat stress study) or provide explanations to measurement outliers.

The use of this alternative model can complement *in vitro* or mathematical systems that are unable to evaluate transgenerational or long-term interactions across whole body organ systems throughout life. Moving forward from this initial evidence supporting the use of this model system for multi-stressor and toxicity testing, additional standardization in culture methodology and reproducibility of parameter evaluation within, as well as across, laboratories needs to be addressed. Additional research on understanding the function of SeMet mechanisms and how SeMet response is affected by maternal age needs to be further investigated. Potential future research areas to address these issues include the role of SeMet in maternal Se transfer, reproductive health, and offspring tolerance to disease and stress during periods of high versus periods of low SeMet exposure.

5 Conclusion

Overall, this study provides evidence that *D. pulex* may be a useful alternative whole animal model for testing multi-stressor interactions across the lifespan. Elevated doses of SeMet exhibited reproductive and developmental toxicity including premature mortality and cessation of reproduction. This study also provided evidence that older maternal age may provide offspring an advantage in heat stress resistance and exasperated the effects of SeMet on time to first reproduction. These effects of maternal age in *Daphnia* at time of offspring development may have a significant effect on the response of offspring to environmental stressors and should be further explored in future studies and in other animal model systems. Ultimately, determination of the utility of *D. pulex* as an alternative model for screening for human toxicity will require additional evaluation of concordance of mechanistic responses.

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Abbreviations

Se	selenium
SeMet	seleno-methionine

NIH	National Institutes of Health
LD50	Lethal Dose for 50% of population
CI	confidence interval
Mdn	median
M	mean
X²	Chi-Square Test

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HIGHLIGHTS

- We evaluated the influence of maternal age on the effects of seleno-methionine (SeMet) on lifespan, reproduction, and heat-stress resistance in *D. pulex*.
- Our results show effects of SeMet-treatment, with the highest SeMet-treatment having reduced lifespan and absence of reproduction.
- An interactive effect between SeMet-treatment and maternal age was found on time to first reproduction.
- Daphnia from late life broods had increased resistance to heat-induced stress.

Maternal Population		Offspring (F1)
Young Maternal Age (early life brood)	X	0-µg SeMet/L
		1-µg SeMet/L
		4-µg SeMet/L
Old Maternal Age (late life brood)	X	0-µg SeMet/L
		1-µg SeMet/L
		4-µg SeMet/L
Measures: Lifespan, Reproduction, Heat stress resistance		

Figure 1.
Maternal Age and SeMet treatment Experimental Design

Figure 2.
Lifespan and Reproduction Experimental Design.

Figure 3.
Heat-Induced Stress Experimental Design.

Figure 4. Kaplan-Meier (survival) curves for each SeMet dose treatment (n=20 in each SeMet treatment group). Based on Log Rank tests, statistically significant differences are present in lifespan between SeMet treatment groups.

Figure 5. Concentration of Se in Daphnia cultured in standard media (selenite control) and 0, 1, and 4- μ g SeMet/L treatments for 12 days (3 replicates per group, 12 individuals in each replicate). Se was not detectable in samples cultured in standard selenite conditions or 0- μ g SeMet/L treatment. Limits of Detection ranged between 101 and 220 ng/g.

Figure 6.

Kaplan-Meier (survival) curve for comparison of early life broods exposed to each SeMet treatment vs. late life broods exposed to each SeMet treatment ($n=25$ per Maternal Age $\times$ SeMet treatment group). $4\text{-}\mu\text{g}$ SeMet/L individuals had significantly shorter lifespans than the lower SeMet treatment groups, but no significant differences were detected between maternal age groups.

Figure 7. Interaction between Maternal Age and SeMet treatment in time to first reproduction. Values represent the mean $\pm$ standard error of the mean (SEM) for onset of reproduction in offspring obtained from a late life brood versus offspring obtained from an early life brood exposed to 0 or 1- μ g SeMet/L SeMet treatments. *Daphnia* exposed to the highest treatment (4- μ g SeMet/L) were excluded from this analysis due to their lack of reproduction throughout life.

Table 1

Summary of statistical results, where SeMet refers to the three SeMet treatments and MA refers to the age of the maternal population at offspring birth.

Variable	SeMet	MA	SeMet × MA	Pattern
Mortality Rate	$p < 0.0001$	$p = 0.66$	$p = 0.44$	The highest treatment (4-μg SeMet/L) mortality rate increased over the lifespan.
Total Reproduction	$p < 0.0001$	$p = 0.32$	$p = 0.44$	The highest treatment (4-μg SeMet/L) resulted in cessation of reproduction
Time to First Reproduction	N/A ([†])	N/A ([†])	$p < 0.0001$	Offspring obtained from older mothers were more susceptible to increased time to first reproduction with SeMet treatment
Survival Post Heat-Induced Stress	$p = 0.12$	$p = 0.002$	$p = 0.10$	Offspring obtained from older mothers had a greater short term survival to heat-induced stress
Presence of Offspring Post Heat Stress	$p = 0.2$	$p = 0.003$	$p = 0.08$	Offspring obtained from older mothers had a greater percent of offspring post heat stress

[†] If an interaction involving a measurement is significant, it is not meaningful to test for the significance of the main effect by itself. Therefore, we denote such cases via N/A.