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STAGE SUSCEPTIBILITY OF JAPANESE MEDAKA (*ORYZIAS LATIPES*) TO SELENOMETHIONINE AND HYPERSALINE DEVELOPMENTAL TOXICITY

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Abstract

Anthropogenic disturbance of seleniferous soils can lead to selenium contamination of waterways. Although selenium is an essential micronutrient, bioaccumulation and maternal transfer of proteinaceous selenomethionine (SeMet) can result in embryo toxicity. Furthermore, as the climate changes, the salinity of spawning grounds in water-restrained estuaries is increasing. Although a small increase in salinity may not directly impact adult fish, it may alter the detoxification strategies of developing organisms. Previous research indicates that hypersalinity may potentiate SeMet embryo toxicity at an early developmental stage. However, embryonic development is a complex, spatiotemporal process with a constantly shifting cellular microenvironment. To generate thresholds and an adverse outcome pathway for the interactions between selenium and salinity, we sought to identify windows of susceptibility for lethality and deformities in the Japanese medaka (*Oryzias latipes*). Embryos were treated in freshwater or saltwater for 24 h with 0.5 μM, 5 μM, and 50 μM SeMet at 6 different developmental stages (9, 17, 25, 29, 34, and 38). Survival, hatch, deformities (total, type, and severity), and days to hatch were quantified. Selenium embryo tissue measurements were performed. Selenomethionine exposures of 5 μM and 50 μM significantly decreased survival and hatch at all stages. However, SeMet uptake was stage-dependent and increased with stage. Stage 17 (early neurulation) was identified as the most susceptible stage for lethality and deformities. Selenomethionine in saltwater caused significantly greater toxicity than freshwater at stage 25 (early organogenesis), suggesting a role for liver and osmoregulatory organogenesis in toxicity.

Keywords

Selenium; Salinity; Climate change; Developmental toxicity; Mixture toxicity

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This article includes online-only Supplemental Data.

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Data availability—Data available upon request from Allison Kupsco at akups001@ucr.edu.

INTRODUCTION

Selenium (Se) is an essential micronutrient that can be toxic to oviparous animals in excess [1]. Present naturally in soils, Se is released into waterways following anthropogenic activity, such as irrigation of arid soils; coal, phosphate and uranium mining; and coal burning in power plants [2–5]. Free Se is often present in water in its inorganic forms of selenate and selenite and can be taken up by microorganisms and converted into various organic forms, including selenomethionine (SeMet) [6]. Dietary uptake has been shown to be the primary route of exposure in fish [7], and the high bioaccumulation factor of Se results in increased exposure to upper trophic levels. Subsequent maternal offloading to eggs can cause embryonic lethality and teratogenesis [8]. The teratogenic effects of Se have been well-characterized in fish and can include spinal, fin, and craniofacial deformities, as well as alterations in organ physiology [9].

Selenium's high bioaccumulation factor confounds water-quality monitoring efforts, and many have criticized free Se water measurements in favor of ovary/egg measurements [10]. Although these tissue-quality measurements are an improvement over water measurements in estimating Se risk, site-specific testing for Se may be necessary, particularly in multiple stressor situations [11]. One common stressor is hypersalinity. As climate change increases salinization of many traditionally fresh waterways [12], the effects of the interaction between SeMet and hypersalinity need to be further elucidated. This issue is particularly relevant in areas such as the San Francisco Bay Delta (CA, USA), where both salinity and Se have been cited as major contributors to declining fish populations [13]. In the San Francisco Bay Delta, rising sea levels leading to increased saltwater intrusion combine with decreased freshwater input to increase salinity [13]. Furthermore, desalination is being considered to meet California's growing drinking water needs, and disposal of desalination brine back into the estuary will further increase salinization. Recent research indicates that hypersaline conditions may potentiate SeMet developmental toxicity; embryos treated with SeMet in saltwater had significantly decreased hatch and increased deformities compared with those treated in freshwater [14,15].

Although toxicity and teratogenicity of Se have been well-characterized in the field [1,7–9], no studies have examined the stage-specific susceptibility of fish to Se. Embryonic development is a complex, spatiotemporal process with constant alterations in the molecular and cellular microenvironment. These changes can modify the mechanism and overall toxicity of a toxicant to the embryo. The identification of critical exposure windows to Se can produce lethal and sublethal thresholds for toxicity and provide additional insight into the mechanism of toxicity for adverse outcome pathway development. Adverse outcome pathways connect a molecular initiating event with a population level outcome via different levels of biological organization [16]. Identifying the critical stage of Se toxicity allows for further elucidation of an adverse outcome pathway for compounds similar to Se and provides a starting point for future studies on mechanisms of SeMet toxicity. Furthermore, this approach is ideal for studying the effects of multiple stressors in development because it allows for isolation of the height of the interaction between Se and hypersalinity.

In the present study, 3 concentrations of waterborne SeMet in freshwater or saltwater were used to model of SeMet exposure to Japanese medaka (*Oryzias latipes*) embryos. Embryos were exposed to SeMet at various stages throughout development. The aims were to determine sensitive developmental stages to SeMet and hypersaline toxicity and to identify thresholds for lethal and sublethal toxicity of SeMet under both freshwater and saltwater conditions using tissue Se values. We hypothesized that earlier developmental stages would be more susceptible to SeMet toxicity than later stages and that hypersalinity would increase SeMet toxicity in a stage-specific manner. These findings allow further elucidation of the mechanism of SeMet embryo toxicity alone and under hypersaline conditions.

METHODS

Animals

Japanese medaka (*O. latipes*) were used for the present study because they are a good euryhaline model organism, are frequently used in toxicity testing, and have well-documented embryonic development. Medaka develop over 10 d to 14 d and hatch as feeding larvae, allowing for isolation of a specific developmental period. Little structural and functional development occurs posthatch. Furthermore, medaka previously have been shown to be sensitive to SeMet and hypersaline toxicity [14,15].

Japanese medaka were cultured at the University of California–Riverside (Riverside, CA, USA) in medium-hard water at 27 °C and were housed in a 2:3 ratio of males to females. They were maintained in a 14:10-h light:dark cycle and fed twice daily a diet of live brine shrimp. Embryos were collected 0 h to 1 h following fertilization, and nonviable embryos were discarded. Oil droplet migration to the vegetal pole was used to determine viable embryos [17].

Overall experimental design

The effects of 3 factors on embryonic development were examined: water type, developmental stage of treatment, and SeMet concentration. To assess these effects, fertilized embryos were placed into dishes containing either freshwater or saltwater. They were monitored throughout development for one of the following stages: 9, 17, 25, 29, 34, or 38. When the embryos had reached the appropriate stage, they were treated with 0 μM, 0.5 μM, 5 μM, or 50 μM SeMet for 24 h. Following exposure, embryos were removed from the SeMet treatment, rinsed, and then replaced into freshwater or saltwater and allowed to develop until hatch. Survival posttreatment, hatch, deformities, and days to hatch were recorded.

Water treatment

To assess the effects of water type, embryos were placed into 60 mm × 15 mm Petri dishes containing either medium-hard fresh water or artificial saltwater from the San Joaquin River that had been prepared in the lab (approximately 10–20 embryos per replicate, 4–6 replicates per treatment). Salts were purchased from Fisher Scientific, and deionized water was obtained via a Milli-Q water purification system (Millipore). Saltwater was prepared based on values collected from Westlands Water District, which is located approximately 10 km

south of Mendota in the San Joaquin River Drainage Basin (CA, USA) [18]. Salinity was 13‰ with 15.21 g/L suspended solids. Although the saltwater used was not embryotoxic, it caused specific tissue death and larval mortality immediately posthatch in 45% percent of replicates, and 60 ± 7.5% of larvae were deformed. The replicates exhibiting these features were discarded and not considered in the analysis.

Developmental stage of treatment

Embryos were treated at 1 of 6 stages—9, 17, 25, 29, 34, or 38—based on Iwamatsu [19]. Stages were spaced regularly throughout development and were chosen to represent key developmental milestones that may be susceptible to selenium and hypersaline toxicity (Table 1). Stage 9 (5 h postfertilization [hpf]), the late morula stage, represents an early exposure window. Stage 17 (25 hpf) is the start of neurulation and was considered based on the well-documented impacts of Se on spinal development [1]. The liver is the primary site of xenobiotic metabolism. Thus, to examine a role for the liver in SeMet and hypersaline toxicity, stage 25 (50 hpf) was examined. Stage 29 (74 hpf) is when the craniofacial cartilage begins to develop [20], and SeMet has been implicated in craniofacial abnormalities [1]. Bone mineralization in medaka occurs at stage 34 (121 hpf) [21]. Finally, stage 38 (192 hpf) occurs just before hatching and is late organogenesis, when full SeMet activation is most likely to occur [19].

Selenomethionine treatment

Once embryos reached the appropriate stage, they were treated with SeMet in freshwater or saltwater (Seleno-l-methionine, purity 98%; Sigma-Aldrich). Although the primary mechanism of environmental embryo exposure is via maternal transfer [6], and thus waterborne exposures do not entirely reflect environmental conditions, they are a simplified method of determining SeMet's effects. Furthermore, free SeMet is the main cause of Se toxicity [6], and precise exposures can be determined with tissue-level analytical chemistry. Although the chorion can function as a barrier against waterborne toxicants, it is water permeable and exhibits varying permeability to organic osmolytes [22]. Hence, we expected SeMet treatments with the chorion to be valid. Embryos were treated with 0.5 μM, 5 μM, and 50 μM SeMet, which correspond to approximately 0.01 mg/L, 0.1 mg/L, and 1 mg/L, respectively. Based on previous work, it is estimated that treatment with 50 μM SeMet for 24 h would be equivalent to the maximum Se tissue concentrations observed in the field following Se contamination [14]. The 5-μM and 0.5-μM SeMet treatments would be indicative of a less impacted environment. Following 24 h of treatment, embryos were replaced into either freshwater or saltwater and allowed to develop until hatch. Water was exchanged every other day. At 24 h posttreatment, survival was assessed and dead embryos were discarded. Embryos were checked daily for hatch and mortality. Dead embryos were removed from the culture dish. Deformities at hatch were recorded for type (cardiac, spinal, craniofacial, fin, or swim bladder). Spinal deformities were categorized as lordosis, scoliosis, and kyphosis. Severity of each deformity was recorded on a scale from 1 to 3. Embryos that had not hatched after 1 mo were considered dead.

Tissue analysis of selenium

Exposures outlined in the *Overall experimental design* section were repeated. Instead of allowing embryonic development to continue posttreatment, live embryos were frozen immediately following the 24-h treatment for analysis with the chorion intact. Because dead embryos may lack the capacity to regulate SeMet uptake, and would thus provide inaccurate measurements, they were not included in the analysis. Not all treatment groups yielded enough live embryos for analysis; therefore, only samples of the lowest concentration demonstrating significance in the survival endpoint were collected. Samples of 40 to 50 embryos were lyophilized and digested in 50% nitric acid at 90 °C for 5 h. Following digestion, samples were diluted to a final nitric acid content of 10%, and Se content was analyzed on an Agilent 7000 inductively coupled plasma-mass spectrometer (ICP-MS) with a detection limit of 0.025 μg/g. Oyster tissue was used for quality control (National Institute of Standards and Technology) with a percent recovery of 98 ± 2.7%.

Statistical analysis

To determine the statistical significance of each factor (water type, SeMet concentration, and developmental stage) and differences between the levels within the factors, 3 different statistical methods were used. Binomial data (nominal data with 2 outcomes; survival, hatch, total deformities, and swim bladder inflation) were analyzed using logistic regression, and pairwise significance was assessed with multiple comparisons. Multinomial data (data with more than 2 nominal outcomes) were assessed with multinomial logistic regressions, and multiple comparisons were performed between the levels of each factor. Multinomial logistic regressions were performed on data reporting the type of deformity and type of spinal deformity; spinal deformities and lordosis were used as reference deformities, respectively. Continuous data (day to hatch, average severity of spinal deformity, and SeMet tissue concentration) were checked for normality and homoscedasticity and were log transformed if they violated these assumptions. Three-way analysis of variance (ANOVA) and Tukey's honest significant difference (HSD) test were used to assess significance. The relationship between Se tissue content and percent deformities was evaluated using linear regression analysis and analysis of covariance (ANCOVA). Data were considered statistically significant at $p \leq 0.05$. All calculations were performed in the statistical program R64 [23].

RESULTS

Survival

Logistic regression analysis indicated that SeMet exposures of 5 μM and 50 μM significantly decreased embryo survival 24 h posttreatment at stages 17 through 38, whereas no differences between controls and 0.5 μM SeMet were observed (Figure 1). When embryos were exposed at stages 17, 25, and 29, survival decreased significantly between 5 μM SeMet and 50 μM SeMet ($p = 0.02$, 0.023 and 0.026, respectively; Figure 1B, C, and D). At stage 9, only 50 μM SeMet significantly decreased survival ($p = 0.005$; Figure 1A). Significant differences were also observed between stages. At 5 μM and 50 μM SeMet, embryos treated at stage 9 had significantly greater survival than all other stages ($p < 0.001$). Saltwater also significantly impacted survival following exposure with 50 μM SeMet ($p = 0.011$), and the

largest difference was observed at stage 25, where survival differed between freshwater (42%) and saltwater (2%) (Figure 1C).

Hatch

Percentage of embryos hatched decreased with increasing concentrations of SeMet (Figure 2). When embryos were treated at stage 17 for 24 h with 0.5 μM SeMet, hatch decreased significantly from controls (logistic regression and multiple comparisons: $p = 0.039$; Figure 2B). Treatments with 5 μM SeMet at all other stages decreased hatch significantly ($p < 0.02$ for all). Significant differences between freshwater and saltwater exposures were observed after 5 μM and 50 μM SeMet ($p = 0.019$ and 0.001, respectively). Consistent with survival percentage, the largest differences in embryo hatch between freshwater and saltwater were recorded at stage 25 (40–50% difference in hatch; Figure 2C). Stage-specific differences in hatch were also observed. After treatment with 5 μM SeMet at stage 17, hatch was significantly less than all other stages ($p < 0.01$ for all). After treatment with 50 μM SeMet, embryos treated at stage 17 had significantly less hatch than all stages except stage 29 ($p < 0.001$). Furthermore, embryos treated with 50 μM SeMet at stage 29 had significantly lower hatch percentage than those treated at stages 9, 34, and 38 ($p = 0.001$, 0.011, and 0.023, respectively).

Deformities

The percentage of total deformities (excluding failed swim bladder inflation) increased significantly with 5 μM SeMet treatment during stages 9, 17, 25, and 34 (logistic regression; $p < 0.025$; Figure 3). Total deformities also increased following treatment with 50 μM SeMet during stages 9 and 25 ($p = 0.003$ and 0.001, respectively; Figure 3A and C). After exposure to 5 μM SeMet, saltwater and freshwater induced significantly different numbers in total deformities ($p = 0.028$), and embryos treated at stage 25 had greater deformities than embryos treated at stages 29 and 34 ($p = 0.035$ and 0.045, respectively). Embryos treated at stages 17 and 25 with 50 μM SeMet also had a significantly greater percentage of deformities than those treated at stage 34 ($p = 0.031$ and 0.013).

Types of deformities

In addition to total deformities, the type of each deformity was also measured as spinal, heart, fin, and cranio-facial. In the multinomial regression model, dose and water type were found to have no significant effect on the type of deformity; however, there were significant differences in types of deformities within and between stages ($p = 0.001$; Figure 4). With a frequency ranging from 60% to 89% of total deformities, embryos exhibited significantly greater spinal deformities than other types of deformities when treated with SeMet at all stages except for stage 9 ($p < 0.01$). Embryos treated at stage 9 had significantly more spinal than fin deformities ($p < 0.001$). Significant differences were also detected between treatments at different stages. Embryos treated at stage 25 had significantly fewer cardiac deformities in relation to spinal deformities than embryos treated at stages 9 ($p < 0.001$), 29 ($p = 0.03$), and 34 ($p = 0.021$). In contrast, embryos treated with SeMet at stage 9 exhibited significantly greater cardiac deformities than those treated at stage 17 ($p = 0.002$) 38 ($p = 0.017$) and significantly greater fin deformities than stages 17 and 34 ($p = 0.033$ and 0.019,

respectively). Embryos exposed at stages 9 and 38 also had greater cranio-facial deformities than those exposed at stage 25 ($p = 0.003$ and 0.03, respectively).

Spinal deformities

Type of spinal deformity was scored as lordosis (inward curvature of the lower spine), kyphosis (rounded outward spinal curvature), or scoliosis (sideways spinal curvature) and assessed via multinomial logistic regression using lordosis as the reference deformity (Supplemental Data, Figure S1). As with type of deformity, stage was the only factor to have a significant effect on the type of spinal deformity in SeMet-treated embryos. Embryos treated at stage 25 had significantly more lordosis than scoliosis or kyphosis (65% compared with 12% and 23% for scoliosis and kyphosis, respectively [$p = 0.001$ and 0.002]). In contrast, embryos treated later, at stage 38, had significantly higher incidence of kyphosis than lordosis ($p = 0.002$). Types of spinal deformities also differed significantly depending on the treatment stage. The embryos treated at stage 25 had significantly less scoliosis and kyphosis than those treated at stage 9 ($p = 0.042$ and 0.022, respectively), 17 ($p = 0.025$ and 0.001, respectively), 29 ($p = 0.005$ and 0.004, respectively), and 38 ($p < 0.001$). Exposure at stage 38 generated significantly greater kyphosis in hatched embryos than embryos treated at stages 9, 17, 25, and 34 ($p = 0.003$, 0.017, 0.001, and 0.04, respectively).

The severity of deformities was also considered using the graduated severity index, and deformities were scored from 1 to 3 [24]. A score of 1 indicated a mild defect in spinal curvature, cranio-facial structure, or mild pericardial edema, whereas scores of 2 and 3 indicated moderate and severe defects, respectively [24]. The sample size for cardiac, cranio-facial, and fin deformities was too small to calculate severity accurately; however, average spinal severities were calculated for each treatment, and significance was determined using a 3-way ANOVA and Tukey's HSD test post hoc (Supplemental Data, Figure S2). No significant differences were observed between freshwater and saltwater; however, a significant interaction between stage, water type, and SeMet dose was detected ($p = 0.001$). Spinal deformities were more severe in embryos treated with 5 μM and 50 μM SeMet than in those treated with 0.5 μM SeMet ($p = 0.011$ and 0.0004, respectively). Spinal deformities in embryos treated at stage 9 were significantly more severe than those treated at stages 34 and 25 ($p < 0.001$). Furthermore, those treated at stage 34 had less severe spinal deformities than embryos treated at stages 9, 17, 29, and 38 ($p = 0.001$, 0.013, 0.021, and 0.018, respectively).

Swim bladder inflation

Although swim bladder inflation is not traditionally considered a deformity, and was thus kept separate from the other deformities measured, the failure of the swim bladder to inflate can impact embryo survival. The percentage of embryos with failed swim bladders following hatch significantly increased following treatment with 5 μM SeMet at stages 9, 17, and 25 (logistic regression; $p = 0.006$, 0.001, and 0.002, respectively); however, none of these differences were found after treatment with 50 μM SeMet (Figure 5A, B and C). At stage 17, embryos treated with 0.5 μM SeMet also had significantly more failed swim bladders ($p = 0.007$). Embryos exposed during stages 9, 17, and 25 had significantly greater incidence of swim bladder failure than those exposed at stage 34 ($p = 0.02$, 0.007, and 0.006,

respectively). Finally, following exposure to 0.5 μM SeMet at stages 17 and 38, embryos had increased swim bladder failure compared with those treated with 0.5 μM SeMet at stage 25 ($p = 0.022$ and 0.039, respectively).

Days to hatch

Selenomethionine dose had little significant effect on the median days to embryo hatch (Supplemental Data, Figure S3). Only embryos treated at stage 34 with 5 μM SeMet took a greater time to hatch than those treated with 50 μM (approximately 13 d compared with 10 d). However, saltwater did significantly decrease the median days to hatch in controls and after 50 μM of treatment ($p = 0.003$ and 0.027, respectively). Furthermore, significant differences in days to hatch were observed between stages. Embryos treated with SeMet at stages 9 and 17 hatched significantly sooner than those treated at stages 25, 29, and 34 ($p < 0.05$ for all).

Tissue Se content

To determine tissue-level Se content, ICP-MS was used to quantify total Se in embryos following the 24-h treatments (Figure 6). Mean control embryo Se content was approximately 3.29 ± 0.24 μg/g. No significant differences were observed between freshwater and saltwater controls at any stage (ANOVA and Tukey's HSD, $p = 0.812$). However, significant differences were observed between treatment concentrations (all $p < 0.001$) and treatment stages (all $p < 0.001$). A dose-dependent increase in Se content was observed for stages 25, 29, 34, and 38 (Figure 6). Embryos assimilated significantly less Se when treated with 5 μM SeMet at stage 9 than stages 25, 29, 34, and 38 (average 5.59 μg/g compared with 24.81 μg/g, 33.89 μg/g, 51.53 μg/g, and 49.52 μg/g, respectively; $p < 0.001$ for all; Figure 6). Embryos treated with 0.5 μM SeMet at stage 17 also assimilated less Se than those treated at stages 29, 34, and 38 (average 5.0 μg/g compared with 16.25 μg/g, 16.51 μg/g, and 15.86 μg/g, respectively; $p \leq 0.001$ for all).

The relationship between mean Se content and average percent deformities was assessed using linear regression (Supplemental Data, Table S1). A significant correlation was detected for stages 25, 29, 34, and 38. An ANCOVA was performed to ascertain differences between stages, and a significant interaction between Se content and stage was detected. Embryos treated at stage 25 had significantly increased deformities by Se content than stages 29, 34, and 38 (i.e., greater slope of regression).

DISCUSSION

Stage sensitivity of Japanese medaka embryos to SeMet and osmotic stress was evaluated using lethal and sublethal indicators. Pairing these endpoints with Se tissue-level measurements allows for the determination of stage-specific thresholds to SeMet under hypersaline conditions, such as those found in the San Francisco Bay Delta. As climate change and agricultural development increase the salinity of the San Francisco Bay Delta, these values become increasingly important for environmental protection. Furthermore, multiple desalination projects are planned for the area, and disposing of desalination brine

back into the estuary may further increase salinity of waterways and concentrate Se within discharges.

Japanese medaka exhibited salinity and stage sensitivity to SeMet at different concentrations. In general, a concentration response to SeMet was observed. However, SeMet concentration was not correlated with embryo Se dose at all stages. For example, embryo Se content was not equal for 5 μM SeMet between stages, although significant differences were observed for all endpoints measured. Significantly less SeMet was assimilated by embryos treated at stages 9 and 17 than at later stages. These differences may be mediated by the chorion. Japanese medaka have a thick (10 μm) chorion, which begins to harden immediately following fertilization, reaching maximum hardness at 6 hpf, then gradually softening again beginning at 6 d postfertilization [25,26]. The chorion may impede aqueous toxicant uptake [27], and a study found that dechorionated Japanese medaka embryos at stage 13 were more sensitive to thiobencarb toxicity than chorionated embryos [28]. Further research is needed to distinguish between the role of the chorion and other mechanisms in aqueous SeMet uptake.

Nevertheless, the waterborne exposures performed in the present study were only used as a model of SeMet exposure; thus, the chorion would not play a role in environmental Se assimilation. In oviparous organisms, exposures to Se in embryos typically occur via maternal transfer [6]. During synthesis, maternal SeMet is incorporated into vitellogenin, a yolk precursor protein, which the embryo then uses throughout development [29]. The efficiency of this process varies greatly by species and reproductive strategy [29]; however, yolk utilization and thus mobilization of SeMet from vitellogenin would be the major determinant of toxicity. Early studies in the medaka have examined transfer of maternal S^{35}-dl-methionine into the yolk and subsequent transfer from the yolk into the embryo [30]. Although no transfer was observed through gastrulation, a rapid increase in radioactivity was detected beginning at stage 19 (closure of the blastopore) and increased steadily until hatch [30], at which point the embryo becomes a feeding larva. Considering that free SeMet is responsible for Se embryo toxicity [6], this evidence suggests that SeMet mobilization from the yolk would occur beginning during the stage 17 treatments performed, but only at the very end of the stage 9 treatments.

Even with differences in Se assimilation caused by chorion interference, stage-specific differences in SeMet toxicity could be observed. Stage 17 appears to be the most sensitive stage for both lethal and sublethal endpoints; significant effects on hatch and deformities were observed at tissue concentrations of 3 μg/g to 6 μg/g Se, which was not significantly different from controls. In addition, although stage 9 appeared less sensitive to higher concentrations of SeMet (significantly greater survival than all other stages treated with 5 μM and 50 μM SeMet), significant deformities and decreased hatch were measured for Se tissue doses of 5 μg/g to 6 μg/g. These values are lower than those reported in the literature. Studies of Northern pike (*Esox lucius*) exposed to mining effluent in Saskatchewan, Canada, found 33.55 μg/g dry weight egg Se to be the 20% effect concentration for deformities [3]. Another study on rainbow trout (*Oncorhynchus mykiss*) exposed to Se in Alberta, Canada, found an egg 15% effect concentration of 21.1 μg/g dry weight Se for skeletal deformities [31]. These reported values are similar to the values following treatment with 5 μM SeMet at

stages 25 and 29 in the present study, which ranged from 23 μg/g to 37 μg/g Se dry weight. However, percentage of population affected at these stages and this treatment level was greater than 15% to 20% and ranged from 40% to 100% for hatch and deformities. These discrepancies may be attributable to differences in species sensitivity or different exposure methods.

Nonetheless, it is expected that earlier developmental stages would be more susceptible to developmental toxicants than later ones. It is generally accepted that susceptibility to teratogenesis follows a specific pattern throughout development. During cleavage and before differentiation, embryos are considered less susceptible to a toxicant, which is expected to either cause mortality or induce sufficient repair [32]. Susceptibility to teratogens is thought to then gradually increase during gastrulation and peak during early organogenesis, then steadily decline until hatch [32]. At stage 9, the embryo is in the early blastula stage, synchronous cleavage is still occurring, and cells are in a single mass. During the 24-h treatment duration, the embryo undergoes gastrulation and neurulation, and somite formation begins [19]. At stage 17, early neurulation begins with the formation of an embryonic body and head. Embryos treated at this stage begin optic, brain, and heart formation during the treatment period [19]. Thus, the increased sensitivity of stage 17 over stage 9 follows the accepted dogma. However, early organogenesis continues through stages 25 and 29, where sensitivity is decreased in relation to Se content. Nonetheless, embryos treated at stage 29 were more susceptible to SeMet toxicity to hatch than those treated at other stages.

In addition to stage-specific effects, the effects of salinity on SeMet toxicity were observed and were stage- and SeMet concentration–specific. Significant differences between freshwater and saltwater were only measured at higher concentrations of SeMet at the 50 μM SeMet treatment level for percentage survival, hatch, and days to hatch and at the 5 μM (approximately 24 μg/g) level for percentage hatch and deformities. These differences were not a result of differences in SeMet uptake between fresh- and saltwater. This indicates that salinity has an increased toxicity at higher levels of SeMet.

Specifically, stage 25 can be identified as particularly sensitive to salinity. Less than 3% of embryos survived or hatched when treated with SeMet in saltwater, in contrast to 40% to 60% in freshwater embryos. Embryos treated with 5 μM SeMet in saltwater also had greater deformities and failed swim bladders than those treated in freshwater (100% compared with 66%). This indicates that saltwater is able to potentiate SeMet toxicity when embryos are treated at stage 25 for 24 h.

These differences may be explained by the developmental changes occurring during this period. Osmoregulation in medaka begins at stage 25 [33], which may influence SeMet toxicity under osmotic stress at this stage. Furthermore, stage 25 is when the liver anlage appears, although full functionality will not be reached until late organogenesis [34]. The liver is the primary site of xenobiotic metabolism. Flavin-containing monooxygenases (FMOs) are located predominantly in the liver during development in mice [35] and are able to oxidize SeMet to the corresponding oxide in mice and humans [36]. Although little work has investigated the role of FMOs during teleost development, FMOs have been shown to be

upregulated during hypersaline conditions in euryhaline fish [14,37,38]. Furthermore, previous research suggests the FMO activation of SeMet can generate oxidative stress and toxicity in Japanese medaka [14]. Developmental expression of FMOs has not been investigated in medaka; however, FMO may play a role in increased SeMet toxicity under hypersaline conditions at stage 25.

The extensive deformities assessment performed in the present study is also highly relevant to SeMet toxicity during osmotic stress. Lemly [1] found a significant exponential correlation between selenium tissue concentrations and percent terata in Se-impacted populations and estimated that 80% of deformed larvae die regardless of their Se concentration. Although this value may vary with the threat of predation, it suggests the importance of considering teratogenesis when evaluating Se toxicity in natural populations. Based on several Se toxicity studies, Lemly [1] developed an index for the impact of teratogenesis at the population level. According to the index, 6% to 25% deformities in a larval population would result in 5% to 20% population mortality, whereas greater than 25% deformities would result in greater than 20% population mortality. This would constitute a major population loss. The present study's results indicate that 5-μg/g to 20-μg/g SeMet exposures at stages 9, 17, and 25 generated significant increases in total deformities of 20% to 80%, which would lead to moderate to severe population mortality.

Type of deformity may also influence the probability of larval survival. For example, spinal deformities can impact swimming performance and thus an animal's ability to obtain food or escape predators. Consistent with previous studies, spinal deformities were the most common type of deformity observed in the SeMet-treated embryos [1,3], and fin deformities were the least common. The specificity of the deformity varied by stage, with embryos treated at stages 17, 25, 29, and 38 having significantly more spinal deformities than any other type. Spinal deformities were further divided into lordosis, kyphosis, and scoliosis. Lordosis is the most prevalent type of spinal deformity caused by Se in the environment, and the present study's results indicate that lordosis was the most common deformity from embryos treated at stage 25. This would suggest that SeMet's mode of action is most specific at this stage.

Pericardial edema was the most prevalent deformity measured in the cardiac category, and edema is generally considered to be a temporary and reversible deformity and is thus not always an accurate measure of teratogenesis past the embryo-larval stage [39]. Conflicting reports on the effects of edema on larvae survival have been reported. Pyron and Beitinger [40] found that fathead minnow larvae from Se treated mothers with edema had low survivability, whereas Hermanutz [41] found no effect of edema on survival in fathead minnow exposed to Se in outdoor streams.

Failed swim bladder is common in aquaculture and has been observed in wild populations at levels of 0.1% to 8% [42]. In aquaculture, failed swim bladder inflation can result in significant losses and has been found to be associated with lordosis [43], which may suggest that not all spinal deformities measured in the present study were caused directly by SeMet. One study found that although fish without swim bladders are able to survive to adulthood, these fish expended more energy to maintain their position in the water column and to catch

prey [44]. Furthermore, Japanese medaka larvae without a swim bladder consumed significantly more oxygen [45]. This information indicates that fish without a swim bladder may have reduced fitness in the wild. Interestingly, salinity also has an impact on swim bladder inflation. In Australian bass (*Mucquaria nouemczculeut*), a decrease in salinity from 25 ppt to 10 ppt resulted in decreased swim bladder inflation [46], whereas the opposite effect was observed in gilthead sea bream [47]. However, there was no significant effect of salinity on swim bladder inflation in the present study.

The present study is the first comprehensive study on the stage-specific effects of SeMet in fish embryos. Our results suggest that earlier stages of SeMet are more susceptible to lethality and deformities. We are further able to identify the peak of hypersaline potentiation of SeMet toxicity at stage 25. This information is valuable for determining site-specific Se thresholds and for characterizing an adverse outcome pathway for selenium in both freshwater and saltwater.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Lemly AD. A teratogenic deformity index for evaluating impacts of selenium on fish populations. Ecotox Environ Safe. 1997; 37:259–266.
2. Outridge PM, Scheuhammer AM, Fox GA, Braune BM, White LM, Gregorich LJ, Keddy C. An assessment of the potential hazards of environmental selenium for Canadian water birds. Environ Rev. 1999; 7:81–96.
3. Muscatello JR, Bennett PM, Himbeault KT, Belknap AM, Janz DM. Larval deformities associated with selenium accumulation in Northern Pike (*Esox lucius*) exposed to metal mining effluent. Environ Sci Technol. 2006; 40:6506–6512. [PubMed: 17120587]
4. Presser, TS., Piper, DZ., Bird, KJ., Skorupa, JP., Hamilton, SJ., Detwiler, SJ., Huebner, MA. Chapter 11: The phosphoria formation: A model for forecasting global selenium sources to the environment. In: James, RH., editor. Handbook of Exploration and Environmental Geochemistry. Vol. 8. Elsevier Science BV; Amsterdam, The Netherlands: 2004. p. 299-319.
5. Ramirez P, Rogers BP. Selenium in a Wyoming grassland community receiving wastewater from an in situ uranium mine. Arch Environ Con Tox. 2002; 42:431–436.
6. Fan TW, The SJ, Hinton DE, Higashi RM. Selenium biotransformations into proteinaceous forms by foodweb organisms of selenium-laden drainage waters in California. Aquat Toxicol. 2002; 57:65–84. [PubMed: 11879939]
7. Phibbs J, Franz E, Hauck D, Gallego M, Tse JJ, Pickering IJ, Liber K, Janz DM. Evaluating the trophic transfer of selenium in aquatic ecosystems using caged fish, X-ray absorption spectroscopy and stable isotope analysis. Ecotox Environ Safe. 2011; 74:1855–1863.
8. Lemly AD. Symptoms and implications of selenium toxicity in fish: The Belews Lake case example. Aquat Toxicol. 2002; 57:39–49. [PubMed: 11879937]
9. Lemly AD. A teratogenic deformity index for evaluating impacts of selenium on fish populations. Ecotox Environ Safe. 1997; 37:259–266.

10. Lemly AD. Guidelines for evaluating selenium data from aquatic monitoring and assessment studies. Environ Monit Assess. 1993; 28:83–100. [PubMed: 24221061]
11. Ohlendorf HM, Covington SM, Byron ER, Arenal CA. Conducting site-specific assessments of selenium bioaccumulation in aquatic systems. Integr Environ Assess Manage. 2011; 7:314–324.
12. Wong, PP., Losada, IJ., Gattuso, JP., Hinkel, J., Khattabi, A., McInnes, KL., Saito, Y., Sallenger, A. Coastal systems and low-lying areas. In: Field, CB.Barros, VR.Dokken, DJ.Mach, KJ.Mastrandrea, MD.Bilir, TE.Chatterjee, M.Ebi, KL.Estrada, YO.Genova, RC.Girma, B.Kissel, ES.Levy, AN.MacCracken, S.Mastrandrea, PR., White, LL., editors. Climate Change 2014: Impacts, Adaptation, and Vulnerability Part A: Global and Sectoral Aspects Contribution of Working Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press; Cambridge, UK: 2014. p. 361-409.
13. Delta Science Council. Final Draft Delta Plan. Delta Stewardship Council; Sacramento, CA, USA: 2013.
14. Lavado R, Shi D, Schlenk D. Effects of salinity on the toxicity and biotransformation of L-selenomethionine in Japanese medaka (*Oryzias latipes*) embryos: Mechanisms of oxidative stress. Aquat Toxicol. 2012; 108:18–22. [PubMed: 22265608]
15. Kupsco A, Schlenk D. Mechanisms of selenomethionine developmental toxicity and the impacts of combined hypersaline conditions on Japanese medaka (*Oryzias latipes*). Environ Sci Technol. 2014; 48:7062–7068. [PubMed: 24856650]
16. Ankley GT, Bennett RS, Erickson RJ, Hoff DJ, Hornung MW, Johnson RD, Mount DR, Nichols JW, Russom CL, Schmieder PK, Serrrano JA, Tietge JE, Villeneuve DL. Adverse outcome pathways: A conceptual framework to support ecotoxicology research and risk assessment. Environ Toxicol Chem. 2010; 29:730–741. [PubMed: 20821501]
17. Kirchen, RV., West, WR. The Japanese medaka: Its care and development. Carolina Biological Supply Company; Burlington, NC, USA: 1976.
18. Schlenk D, Zubcov N, Zubcov E. Effects of salinity on the uptake, biotransformation, and toxicity of dietary seleno-L-methionine to rainbow trout. Toxicol Sci. 2003; 75:309–313. [PubMed: 12857940]
19. Iwamatsu T. Stages of normal development in the medaka *Oryzias latipes*. Mech Develop. 2004; 121:605–618.
20. Langille RM, Hall BK. Development of the head skeleton of the Japanese medaka, *Oryzias latipes* (Teleostei). J Morphol. 1987; 193:135–158.
21. Yasutake J, Inohaya K, Kudo A. *Twist* functions in vertebral column formation in medaka, *Oryzias latipes*. Mech Develop. 2004; 121:883–894.
22. Alderdice, DF. Osmotic and ionic regulation in teleost eggs and larvae. In: Hoar, WS., Randall, DJ., editors. Fish Physiology: The Physiology of Developing Fish Eggs and Larvae. Vol. 11. Academic Press; San Diego, CA, USA: 1988. p. 163-251.
23. R Core Team. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing; Vienna, Austria: 2012.
24. Holm, J., Palace, VP., Wautier, K., Evans, RE., Baron, CL., Podemski, C., Siwik, P., Sterling, G. An assessment of the development and survival of wild rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*) exposed to elevated selenium in an area of active coal mining. Proceedings, 26th Annual Larval Fish Conference; Institute of Marine Research, Bergen, Norway. July 22–27, 2003; 2003. p. 257-273.
25. Suga N. Changes of the toughness of the chorion of fish eggs. Embryologia. 1963; 8:63–74.
26. Yamagami K, Hamazaki TS, Yasumasu S, Masuda K, Iuchi I. Molecular and cellular basis of formation, hardening, and breakdown of the egg envelope in fish. Int Rev Cytol. 1992; 136:51–92. [PubMed: 1506146]
27. Blaxter, JHS. Pattern and variety in development. In: Hoar, WS., Randall, DJ., editors. Fish Physiology. Vol. XI. Academic Press; San Diego, CA, USA: 1988. p. 1-58.
28. Villalobos SA, Hamm JT, Teh SJ, Hinton DE. Thiobencarb-induced embryotoxicity in medaka (*Oryzias latipes*): Stage-specific toxicity and the protective role of chorion. Aquat Toxicol. 2000; 48:309–326. [PubMed: 10686335]

29. Janz, DM., DeForest, DK., Brooks, ML., Chapman, PM., Gilron, G., Hoff, D., Hopkins, WA., McIntyre, DO., Mebane, CA., Palace, VP., Skorupa, JP., Wayland, M. Selenium toxicity to aquatic organisms. In: Chapman, PM.Adams, WJ.Brooks, ML.Delos, CG.Luoma, SN.Maher, WA.Ohlendorf, HM.Presser, TS., Shaw, DP., editors. Ecological Assessment of Selenium in the Aquatic Environment. SETAC; Pensacola, Florida, USA: 2010. p. 139-240.
30. Monroy A, Ishida M, Nakano E. The pattern of transfer of the yolk material to the embryo during the development of the teleostean tish, *Oryzias latipes*. Embryologia. 1961; 6:151–158.
31. Holm J, Palace VP, Siwik P, Sterling G, Evans RE, Baron CL, Werner J, Wautier K. Development effects of bioaccumulated selenium in eggs and larvae of two salmonid species. Environ Toxicol Chem. 2005; 24:2373–2381. [PubMed: 16193768]
32. Timbrell, JA. Principles of Biochemical Toxicology. 4th. Informa Healthcare; London, UK: 2009. Toxic responses to foreign compounds; p. 193-287.
33. Thermes V, Lin CC, Hwang PP. Expression of Ol-foxi3 and Naþ/Kþ-ATPase in ionocytes during the development of euryhaline medaka *(Oryzias latipes*) embryos. Gene Expr Patterns. 2010; 10:185–192. [PubMed: 20388555]
34. Hinton DE, Wakamatsu Y, Ozato K, Kashiwada S. Imaging liver development remodeling in the see-through medaka fish. Comparative Hepatology. 2004; 3:30–34.
35. Janmohamed A, Hernandez D, Phillips IR, Shepard EA. Cell-, tissue-, sex- and developmental stage-specific expression of mouse flavin-containing monooxygenases (Fmos). Biochem Pharmacol. 2004; 68:73–83. [PubMed: 15183119]
36. Krause RJ, Glocke SC, Sicuri AR, Ripp SL, Elfarra AA. Oxidative metabolism of seleno-L-methionine to L-methionine selenoxide by flavin-containing monooxygenases. Chem Res Toxicol. 2006; 19:1643–1649. [PubMed: 17173378]
37. Schlenk D, Peters LD, Livingstone DR. Correlation of salinity with flavin-containing monooxygenase activity but not cytochrome P450 activity in the euryhaline fish (*Platichthys* fl*esus*). Biochem Pharmacol. 1996; 52:815–818. [PubMed: 8765480]
38. El-Alfy A, Larsen B, Schlenk D. Effect of cortisol and urea on flavin monooxygenase activity and expression in rainbow trout, *Oncorhynchus mykiss*. Mar Environ Res. 2002; 54:275–278. [PubMed: 12408576]
39. Lemly AD. Teratogenic effects of selenium in natural populations of freshwater fish. Ecotox Environ Safe. 1993; 26:181–204.
40. Pyron M, Beitinger TL. Effect of selenium on reproductive behavior and fry of fathead minnows. B Environ Contam Tox. 1989; 42:609–613.
41. Hermanutz RO. Malformation of the fathead minnow (*Pimephales promelas)* in an ecosystem with elevated selenium concentrations. B Environ Contam Tox. 1992; 49:290–294.
42. Egloff M. Failure of swim bladder of perch, *Perca fluviatilis* L. found in natural populations. Aquat Sci. 1996; 58:15–23.
43. Chatain B. Abnormal swimbladder development and lordosis in sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus auratus*). Aquaculture. 1994; 119:371–379.
44. Czesny SJ, Graeb BDS, Dettmers JM. Ecological consequences of swimbladder noninflation for larval yellow perch. T Am Fish Soc. 2005; 134:1011–1020.
45. Marty GD, Hinton DE, Cech JJ. Oxygen consumption by larval Japanese medaka with inflated or noninflated swim bladders. T Am Fish Soc. 1995; 124:623–627.
46. Battaglene SC, Talbot RB. Initial swim bladder inflation in intensively reared Australian bass larvae. *Macquaria novemacctleata* (Steindachner) (Perciformes: Percichthyidae). Aquaculture. 1990; 86:431–442.
47. Woolley LD, Qin JG. Swim bladder inflation and its implication to the culture of marine fish larvae. Aquaculture. 2010; 2:181–190.

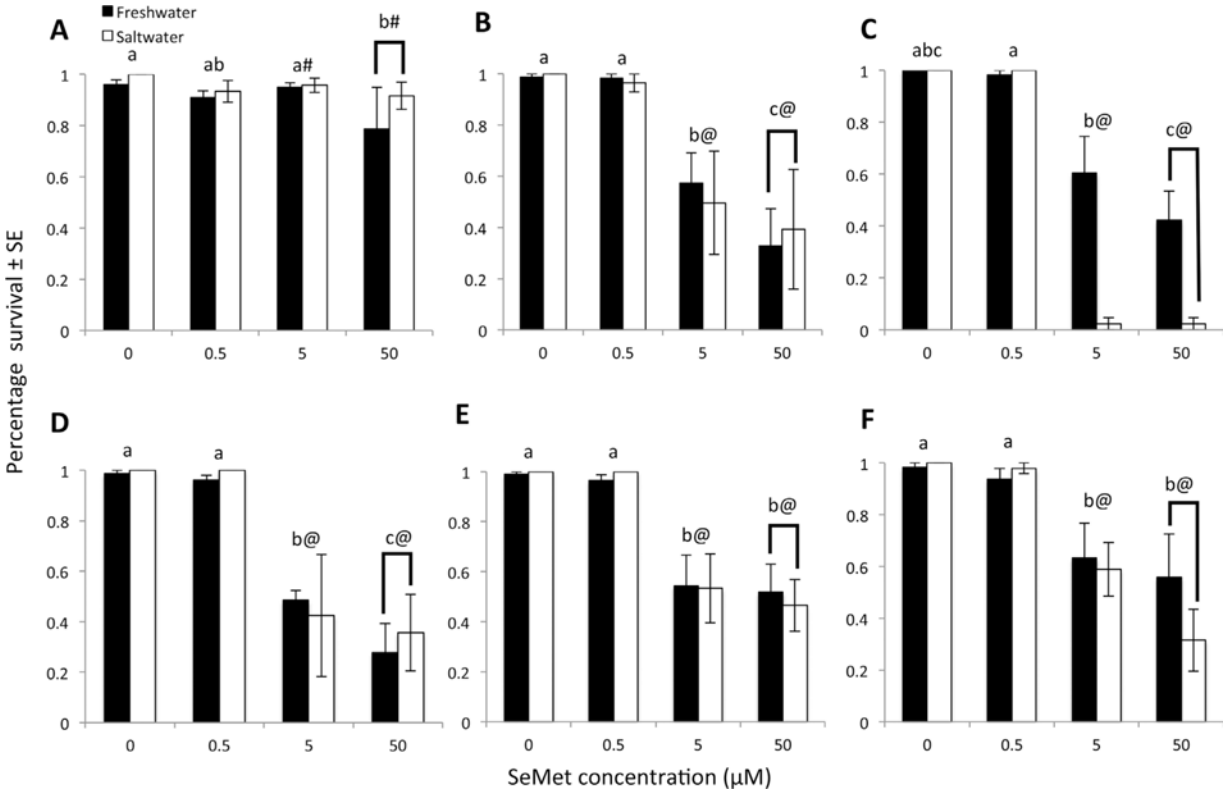

Figure 1.

Percentage survival ± standard error (SE) of embryos 24 h post 24-h treatment with 0.5 μM, 5 μM, and 50 μM selenomethionine (SeMet) in either freshwater (black bars) or saltwater (white bars) (n = 3–6). Each graph represents a stage of treatment: (**A**) stage 9, (**B**) stage 17, (**C**) stage 25, (**D**) stage 29, (**E**) stage 34, and (**F**) stage 38. Statistical significance was assessed using a logistic regression followed by multiple comparisons, and $p \leq 0.05$ was considered statistically significant. Differing letters represent significant differences between concentrations at a specific stage, and forks denote differences between fresh and saltwater at a specific dose. Symbols (#, @) represent significant differences between stages (different graphs) at specific doses; for example, the percentage survival at 5 μM at stage 9 with the # (**A**) is significantly different than the percentage survival at 5 μM with the @ in stages 17 through 38 (**B**–**F**).

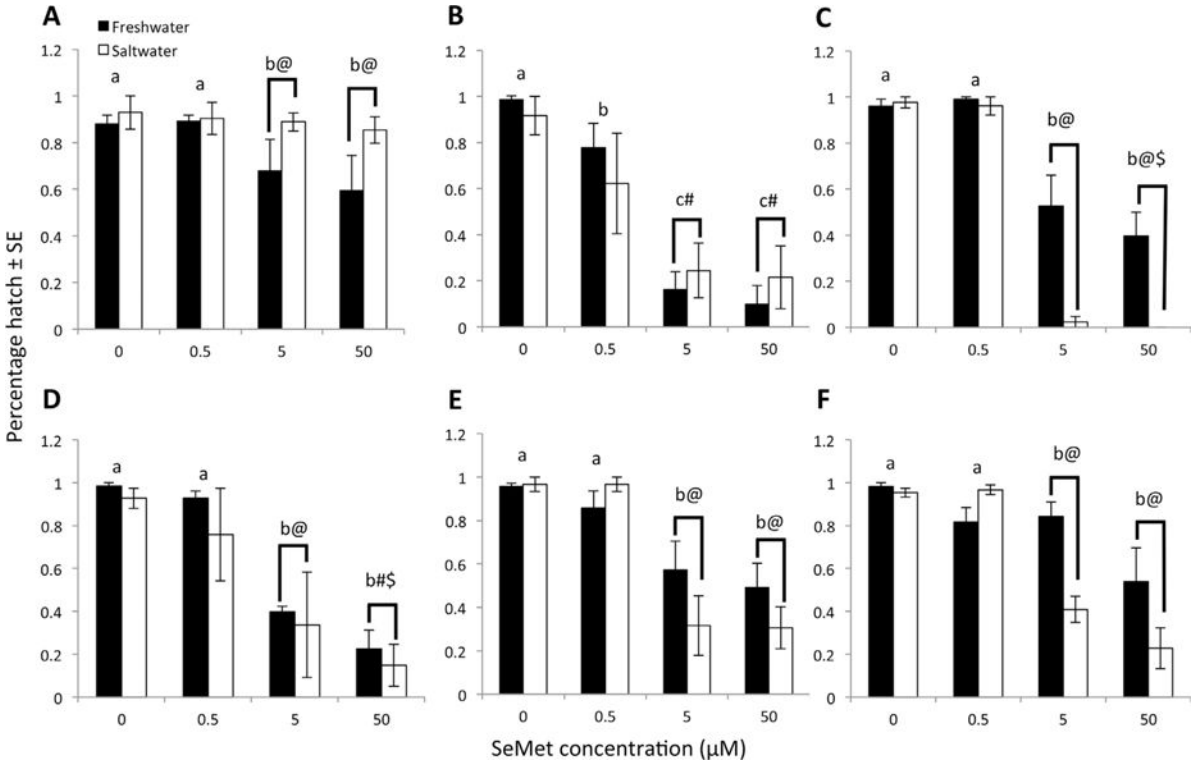

Figure 2.

Percent hatch ± standard error (SE) following 24 h of treatment with 0.5 μM, 5 μM, and 50 μM selenomethionine (SeMet) in either freshwater (black bars) or saltwater (white bars) (n = 3–6). Each graph represents a stage of treatment: (**A**) stage 9, (**B**) stage 17, (**C**) stage 25, (**D**) stage 29, (**E**) stage 34, and (**F**) stage 38. Statistical significance was assessed using a logistic regression followed by multiple comparisons, and $p \leq 0.05$ was considered statistically significant. Differing letters represent significant differences between concentrations at a specific stage, and forks denote differences between freshwater and saltwater at a specific concentration. Symbols (#, @) represent significant differences between stages (different graphs) at specific doses.

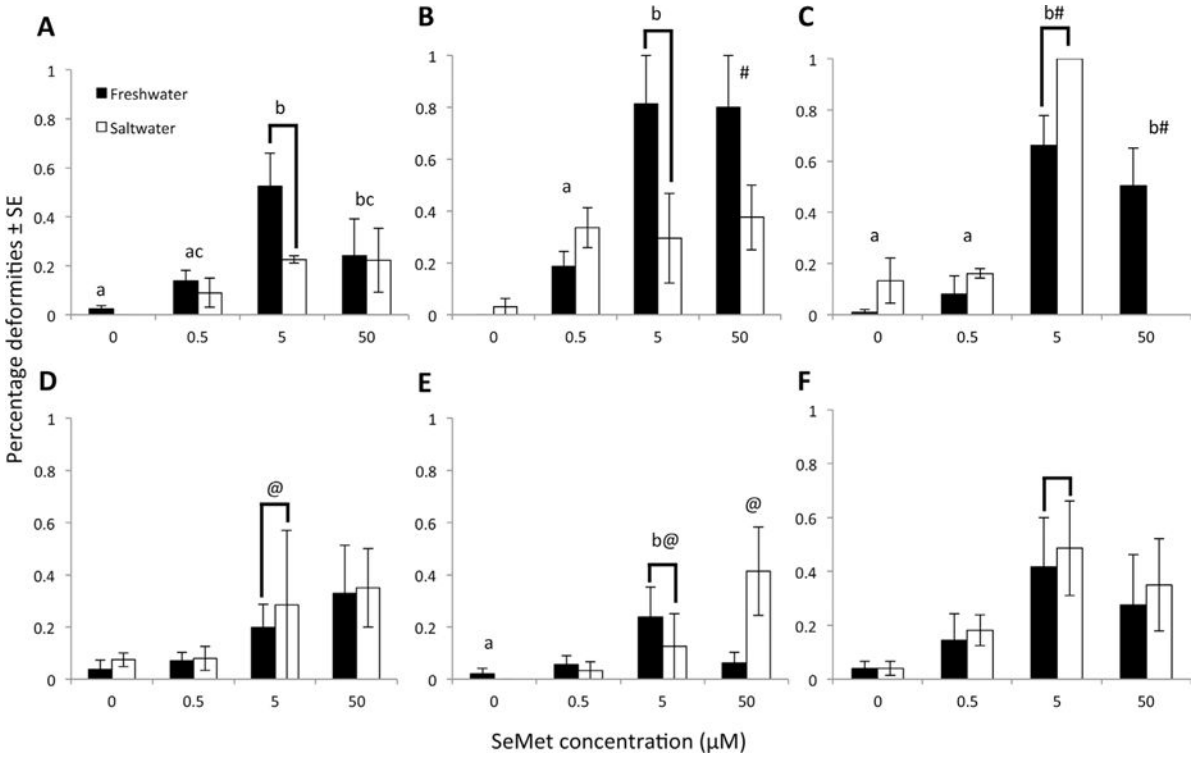

Figure 3.

Percent deformities ± standard error (SE) following 24 h of treatment with 0.5 μM, 5 μM, and 50 μM selenomethionine (SeMet) in either freshwater (black bars) or saltwater (white bars) (n = 3–6). Each graph represents a stage of treatment: (**A**) stage 9, (**B**) stage 17, (**C**) stage 25, (**D**) stage 29, (**E**) stage 34, and (**F**) stage 38. Statistical significance was assessed using a logistic regression followed by multiple comparisons, and $p \leq 0.05$ was considered statistically significant. Differing letters represent significant differences between concentrations at a specific stage, and forks denote differences between fresh and saltwater at a specific concentration. Symbols (#, @) represent significant differences between stages (different graphs) at a specific concentration.

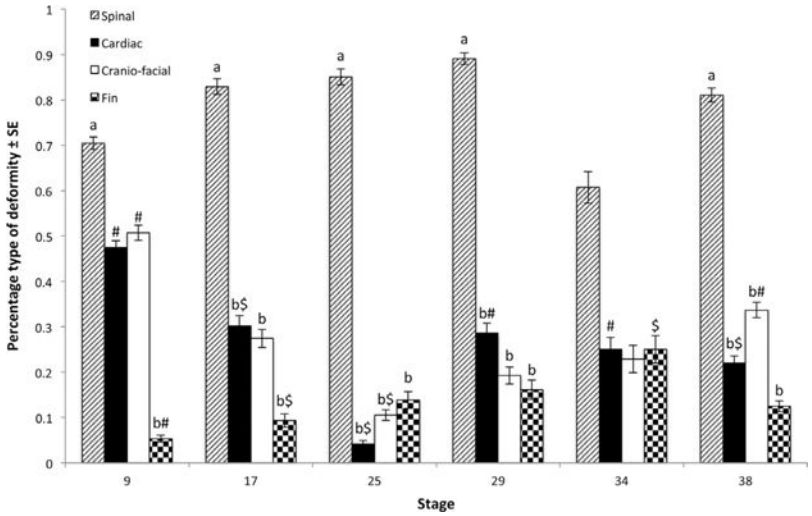

Figure 4.

Percentage of each type of deformity inflation ± standard error (SE) following 24 h of treatment with all concentrations of selenomethionine (SeMet) and both types of water (n = 14–24 replicates with 10–20 embryos per replicate). Types of deformities measured include: spinal (lined bars), cardiac (black bars), cranio-facial (white bars), and fin (checkered bars). Statistical significance was assessed with a multinomial logistic regression using spinal deformities as the reference. $P \leq 0.05$ was considered statistically significant. Differing letters represent significant differences within a stage, and symbols (#, $) represent significant differences among 1 type of deformity between stages.

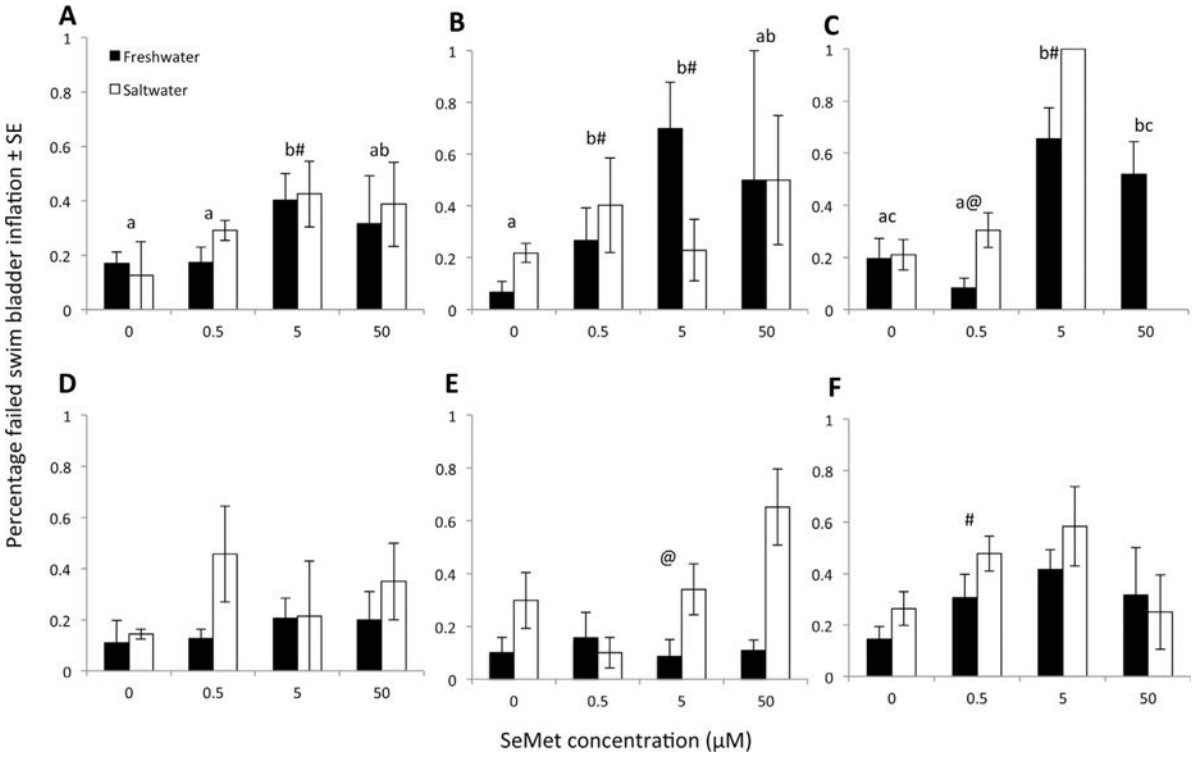

Figure 5.

Percent failed swim bladder inflation ± standard error (SE) following 24 h of treatment with 0.5 μM, 5 μM, and 50 μM selenomethionine (SeMet) in either freshwater (black bars) or saltwater (white bars) (n = 3–6). Each graph represents a stage of treatment: (**A**) stage 9, (**B**) stage 17, (**C**) stage 25, (**D**) stage 29, (**E**) stage 34, and (**F**) stage 38. Statistical significance was assessed using a logistic regression followed by multiple comparisons, and $p \leq 0.05$ was considered statistically significant. Differing letters represent significant differences between concentrations at a specific stage, and forks denote differences between fresh and saltwater at a specific concentration. Symbols (#, @) represent significant differences between stages (different graphs) at specific concentrations.

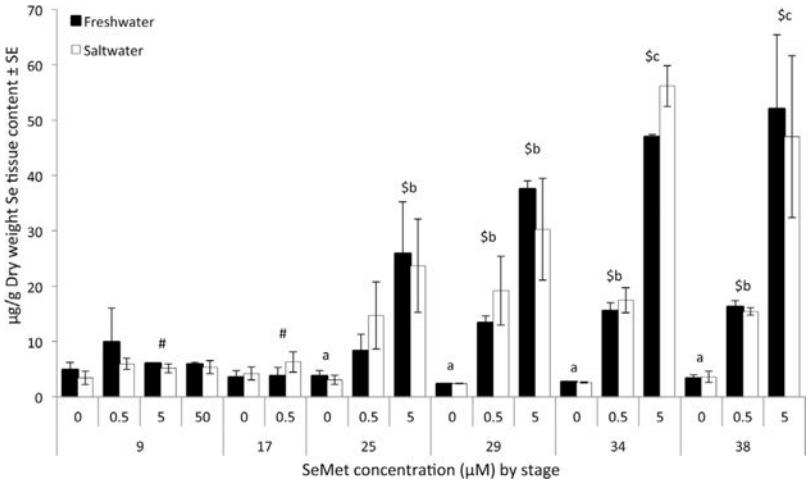

Figure 6.

Whole egg Se content (µg/g dry wt) in embryos surviving following 0.5 µM, 5 µM, and 50 µM selenomethionine (SeMet) treatment in either freshwater (black bars) or saltwater (white bars) ($n = 3$). Samples were only collected for SeMet concentrations where the lowest level of significance was observed in the apical endpoints. Statistical significance was assessed with a 3-way analysis of variance followed by a Tukey's honest significant difference test post hoc, and $p \leq 0.05$ was considered statistically significant. Differing letters represent significant differences between concentrations at a specific stage, and differing symbols (#, $) denote differences stages for a specific concentration.

Table 1

Developmental stages of medaka development chosen for 24 h of selenomethionine treatment

Stage	Hours postfertilization	Developmental milestone
9	5	Late morula
17	25	Early neurulation
25	50	Liver anlagae
29	74	Cranio-facial cartilage
34	121	Bone mineralization
38	192	Late organogenesis