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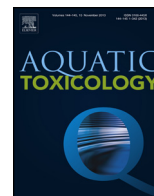
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Parental dietary seleno-L-methionine exposure and resultant offspring developmental toxicity

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ABSTRACT

Selenium (Se) leaches into water from agricultural soils and from storage sites for coal fly ash. Se toxicity causes population and community level effects in fishes and birds. We used the laboratory aquarium model fish, Japanese medaka (*Oryzias latipes*), an asynchronous breeder, to determine aspects of uptake in adults and resultant developmental toxicity in their offspring. The superior imaging properties of the model enabled detailed descriptions of phenotypic alterations not commonly reported in the existing Se literature. Adult males and females in treatment groups were exposed, separately and together, to a dry diet spiked with 0, 12.5, 25, or 50 $\mu\text{g/g}$ (dry weight) seleno-L-methionine (SeMet) for 6 days, and their embryo progeny collected for 5 days, maintained under controlled conditions and observed daily for hatchability, mortality and/or developmental toxicity. Sites of alteration included: craniofacial, pericardium and abdomen (Pc/Ab), notochord, gall bladder, spleen, blood, and swim bladder. Next, adult tissue Se concentrations (liver, skeletal muscle, ovary and testis) were determined and compared in treatment groups of bred and unbred individuals. No significant difference was found across treatment groups at the various SeMet concentrations; and, subsequent analysis compared exposed vs. control in each of the treatment groups at 10 dpf. Increased embryo mortality was observed in all treatment groups, compared to controls, and embryos had a decreased hatching rate when both parents were exposed. Exposure resulted in significantly more total altered phenotypes than controls. When altered phenotypes following exposure of both parents were higher than maternal only exposure, a male role was suggested. The comparisons between treatment groups revealed that particular types of phenotypic change may be driven by the sex of the exposed parent. Additionally, breeding reduced Se concentrations in some adult tissues, specifically the liver of exposed females and skeletal muscle of exposed males. Detailed phenotypic analysis of progeny from SeMet exposed parents should inform investigations of later life stages in an effort to determine consequences of early life exposure.

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1. Introduction

Selenium (Se), an essential micronutrient that maintains physiological homeostasis (Janz, 2012), can result in developmental toxicity when present in excess. Particularly affected are yolk-producing species, such as birds and fishes (Rigby et al., 2014). Hazardous Se exposures are widespread and arise primarily from anthropogenic practices (Janz, 2012; Santos et al., 2015). For example, in the Kesterson Wildlife Refuge, CA, the irrigation of seleniferous soils resulted in leaching and high Se concentrations

in evaporative ponds and drains (Hamilton, 2004; Saiki and Lowe, 1987). Field studies showed severe embryotoxicity in black-necked stilts (*Himantopus mexicanus*), American coots (*Fulica americana*), and western mosquitofish (*Gambusia affinis*) (Hamilton, 2004; Ohlendorf et al., 1986; Saiki and Ogle, 1995). In Belews Lake, NC, offsite waste from a coal-fired power plant caused high concentrations of Se (Cumbie and van Horn, 1978; Hamilton, 2004; Lemly, 2002a,b). Fish toxicity appeared as reduced viable egg production, increased post-hatch mortality, and elimination of 19 of 20 resident species (Lemly, 2002a,b). The discharge of Se-rich fly ash effluent into Martin Lake, TX was followed by liver and ovarian alterations with reduced reproductive success in adult redear sunfish (*Lepomis microlophus*) (Sorensen, 1988). Coal ash spills in Kingston, TN and more recently in the Dan River, NC released 4.1 million m^3 and

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39,000 tons of Se-rich coal ash, respectively, threatening birds and fish in those ecosystems (Lemly, 2015; Mathews et al., 2014).

Se exists in various forms, with the three primary waterborne species being selenate, selenite, and organoselenide (e.g., selenomethionine). Inorganic Se (selenate and selenite) is commonly released from geologic or anthropogenic sources (USEPA, 2014), dissolves in surface waters, is taken up by aquatic plants and bacteria, and is incorporated in tissues and converted to organoselenium via a methionine replacement (Schrauzer, 2000; USEPA, 2014). Selenomethionine (SeMet), the major form of organic Se in the aquatic food chain, is readily absorbed by fish through diet and is considered to be the primary form causing ecotoxicity (Chapman et al., 2010). Its incorporation into aquatic food webs leads to bioaccumulation and in some cases biomagnification (Fan et al., 2002; Janz, 2012; Rigby et al., 2014). SeMet is more toxic than inorganic Se to developing offspring (Woock et al., 1987), making embryotoxicity the major problem and forming our rationale for its selection in this study.

Maternal transfer involves shuttling of liver-derived vitellogenin precursors to maturing eggs during vitellogenesis via a Se-sulfur substitution followed by assimilation in the developing embryo (Janz et al., 2010). Although hatchability of embryos is generally not affected (Hamilton, 2004; Woock et al., 1987), deformities become evident with development, impairing critical body functions (Lemly, 1997a,b,c, 2002a,b). In medaka (*Oryzias latipes*), maternal transfer was followed by use of ⁷⁵Se to determine rapid movement of dietary Se to ovary and its maturing eggs (Conley et al., 2014). To date, most field and laboratory Se toxicity studies concentrate on maternal exposure. Why include males? Se toxicity in paternal fish is virtually unknown. However, in field studies, elevated testicular Se, relative to other tissues, has been reported (Table 1), but the significance of this on offspring has not been determined.

Laboratory aquatic model fish, such as zebrafish (*Danio rerio*), medaka, and fathead minnow (*Pimephales promelas*) are important research tools enabling manipulation of environmental conditions (Cheng et al., 2012). They provide for careful control of exposure levels of toxicants in food and water while transparent chorions enable detailed observations and evaluation of development (Iwamatsu, 2004; Takeda and Shimada, 2010; Westerfield, 2000). Recent Se dietary studies have used these models to good advantage (Conley et al., 2014; Thomas and Janz, 2014). Because these species breed asynchronously, the timing of exposure is important. In medaka, the process of vitellogenesis and associated oocyte maturation of individual follicles requires 72 h (Kinoshita et al., 2009; Shibata et al., 2011). During this time, immediate diet supplies nutrients and trace elements to maturing oocytes, underscoring the linkage of recent dietary consumption to observable results in short time scales (Conley et al., 2014).

Our objective was to investigate how parental (maternal, paternal, or both) dietary SeMet exposure affected medaka embryo viability and development. It was important to understand the type and incidence of developmental abnormalities, some of which have not been reported in previous studies, as they related to dietary SeMet exposure and the sex of the exposed parent. This study is unique because, by using a laboratory aquatic model to vary SeMet concentrations while exposing one or both parents, we were able to deepen our understanding of Se toxicity in offspring.

2. Materials and methods

2.1. Medaka culture

Our breeding colony of orange-red (OR) medaka was maintained at Duke University under animal care and maintenance protocols approved by the Institutional Animal Care and Use Com-

mittee. Adult fish were maintained in closed recirculating water conditions at 24 °C under a 14:10 light:dark cycle, fed three times per day with Otohime β1 commercial dry diet (200–360 μm, Pentair Aquatic Eco-Systems, Apopka, FL, USA) and supplemented twice daily with *Artemia* nauplii (Pentair Aquatic Eco-Systems, 90% Great Lakes Strain). Our colony has been maintained for over 15 years and has a hatch rate of 85% or higher. Medaka from this colony have been used extensively in characterizing the liver, testis, and ovary including histopathology, and growth and development (Davis et al., 2002; Koger et al., 1999; Miller et al., 2012).

2.2. Diet preparation

Selenium test concentrations and exposure duration were based on preliminary feeding trials with female medaka. SeMet concentrations of 8, 20, 50, and 200 μg/g dry weight (dw) were tested and egg production and resultant deformities recorded. The 8 μg/g diet proved unsatisfactory with <5% deformed embryos. The 20 μg/g diet was the most reproducible with ≥33% deformed embryos. The 50 and 200 μg/g diets resulted in increased instances of small, nonviable embryonated eggs. Therefore, we chose a 25 μg/g dw SeMet concentration for further testing, halving and doubling it to achieve a range of concentrations. In keeping with the 72 h oogenesis (Kinoshita et al., 2009; Shibata et al., 2011), exposure durations of 5, 7, and 10 days were tested. Fish exposed for 10 days were less likely to breed, failing to produce egg clutches for the first 2–3 days or altogether. The 5 and 7 day exposure periods were satisfactory and accordingly a 6 day duration was chosen.

Dry diets with 12.5, 25, and 50 μg/g dw seleno-L-methionine (Sigma-Aldrich, St. Louis, MO) were prepared. First, a stock solution was made containing 1 mg/mL of SeMet in MilliQ water (Millipore, USA). Then 20 mL solutions of the desired concentrations of SeMet were prepared via serial dilutions of this stock solution. Each was poured over 20 g of dry food in a glass Petri dish and thoroughly mixed with a spatula to ensure uniform distribution of the liquid. The mixture was then spread along the bottom of the dish and scored with a tapered spatula to increase surface area and promote drying. Prepared dishes were then placed on a bed of Drierite (Sigma-Aldrich) in an enclosed container at 4 °C. Drierite was recharged every 1–2 days or as needed and food was broken apart and stirred daily, exposing additional surface area for drying. Total drying time was 1 week when food particles were at or very near their original size. Diet was stored in these same containers until the time of feeding to prevent moisture accumulation.

2.3. Parental exposure regime

The following exposure regime was repeated for each of the SeMet test concentrations and embryo collections. Adult male and female medaka of reproductive age (6–9 months) were randomly selected from our breeding colony. Reproductive status of individuals was determined by isolating together a male and female and observing their tank for 1 week for viable embryos. Individuals deemed fertile were transferred to the laboratory and maintained under natural ambient winter lighting of 9:15 light:dark at 24 °C. Groups of males (5/tank) and females (3/tank) were isolated from each other in a series of 3 L AHAB semi-static tanks (Fig. S1A) containing 1.5 L of 0.1% (w/v) aquarium salt in MilliQ water that had been oxygenated with an air stone for at least 24 h. Individuals from each group were assigned to one of four treatments: (1) females exposed, males unexposed; (2) females unexposed, males exposed; (3) females exposed, males exposed, and (4) females unexposed, males unexposed (control). Each treatment group was repeated in triplicate (Fig. S1A).

Before beginning the experiment, fish were allowed to acclimate to these conditions for 3 days while following the breeding colony

Table 1
Selenium concentrations in male tissues.

Species	Exposure duration	Wet/dry weight	Testes-reference/control (μg/g)	Testes-exposure (μg/g)	Muscle/carcass-reference/control (μg/g)	Muscle/carcass-exposure (μg/g)	Reference
Bluegill (<i>Lepomis macrochirus</i>)	Field collection	Wet weight	N/A	15.2	N/A	17.8	Cumbie and Van Horn (1978)
Redear sunfish (<i>Lepomis microlophus</i>)	Field collection	Wet weight	N/A	22.8	N/A	36.5	
Bluegill (<i>Lepomis macrochirus</i>)	Field collection	Wet weight	0.5 ^{a,b}	3.35 ^b	0–0.5 ^{a,b}	4.64 ^b	Baumann and Gillespie (1986)
Largemouth bass (<i>Micropterus salmoides</i>)	Field collection	Wet weight	<0.1 ^{a,b}	2.38 ^b	0–0.5 ^{a,b}	2.63 ^b	
Razorback suckers (<i>Xyrauchen texanus</i>)	Field collection	Dry weight	N/A	<1.1–6.7 ^c	N/A	3.55–25.95	Hamilton and Waddell (1994)
Fathead minnow (<i>Pimephales promelas</i>)	105 Days (30 ppm)	Dry weight	3.85 ± 2.10	7.82 ± 1.10	1.93 ± 0.51	8.77 ± 1.22	Ogle and Knight (1989)
Bluegill (<i>Lepomis macrochirus</i>)	258 Days (10 μg/L)	Wet weight	0.6 ± 0.4	3.0 ± 1.5	0.3 ± 0.2	1.8 ± 0.8	Hermanutz et al. (1992)
	258 Days (30 μg/L)	Wet weight	0.6 ± 0.4	6.3 ± 0.4		2.8 ± 0.6	
	356 Days (μg/L)	Wet weight	1.2 ± 0.9	7.6 ± 1.5	0.3 ± 0.2	4.2 ± 1.6	
Bluegill (<i>Lepomis macrochirus</i>)	60 Days (33 μg/g + 11 μg/L)	Dry weight	0.3 ^a	36 ^a	0.1 ^a	16 ^a	Coyle et al. (1993)
	140 Days (33 μg/g + 11 μg/L)	Dry weight	0.3 ^a	32 ^a	0.1 ^a	18 ^a	

Note: μg/g indicates dietary, μg/L indicates aqueous exposure.

^a Value approximated from figure.

^b Means pooled from all cooling reservoirs.

^c Milt first three studies test muscle, the last two test carcass; all muscle/carcass concentrations are samples from males except for Hermanutz et al. (1992).

feeding schedule of 3 times per day with *Artemia* nauplii supplemented during the first 2 feedings. After the acclimation period, fish were fed according to this schedule for a period of 6 days, totaling approximately 1% of body weight per individual per day of their assigned spiked or control food; however, the amount of *Artemia* nauplii solution was reduced to 3 drops per supplementation to ensure complete ingestion of the dry food.

After the 6 day exposure period, adults were gently removed from their tanks and rinsed, the tanks thoroughly cleaned, and complementary sexes mixed into breeding groups according to their previously assigned treatment groups (Fig. S1B). During this breeding period, fish remained on the same feeding schedule and returned to control diet with the amount of *Artemia* nauplii solution increased to back to normal to encourage egg production.

Tank maintenance included daily siphoning to reduce waterborne accumulation of SeMet from fish waste, volumes of water replaced were up to 1.5 L, resulting in ~30% water change. Adults were observed for normal activity, feeding and any signs of stress.

2.4. Offspring collection and observation

After cessation of the adult dietary exposure, embryonated eggs were collected for 5 days (Fig. S2) by siphoning from the bottom of tanks approximately 20 min after each feeding or collected directly as egg masses at the end of each day. Embryonated eggs were gently rolled on moistened paper towels to separate and clean them and then transferred to Petri dishes (VWR, Corning) labeled with collection date and tank number. Unfertilized and fertilized embryos were counted and the former discarded. Dishes containing 0.1% (w/v) artificial salt in MilliQ water were refreshed daily and maintained in a 26 °C incubator with a 14:10 light:dark cycle, on a slowly moving orbital shaker, until hatch.

Individuals in each dish were examined daily under a stereomicroscope (Nikon SMZ1500, Melville, NY, USA) for development according to Iwamatsu (2004) and Kinoshita et al. (2009). From a compilation of our preliminary trial, investigations in our laboratory and selected studies from the literature (Dong et al., 2012, 2013, 2014; Holm et al., 2005; Lemly, 1997a,b,c; Macaulay et al., 2015; Villalobos et al., 2000), phenotypic responses were formulated as pre-defined categories to evaluate embryo alterations using a simple frequency analysis (presence/absence). These categories included: *mortality*; *hatching time/success*; *craniofacial abnormality*, change in size and/or shape of the jaw and specifically *ocular deformities*, agenesis, edema, asymmetry, pigment loss or irregularities; *pericardial and abdominal (Pc/Ab) edema*, swelling of pericardial cavity (Pc) and/or a clear space between the long axis of the body and the dorsal margin of the yolk sac (Ab); *notochord malformation*; *spleen color*, lack or change in color; *gall bladder color*, lack or change in color; *blood pooling*; and, at or after hatch, *swim bladder noninflation*, partially inflated or uninflated in post-hatch individuals; *other altered phenotypes*, i.e., not listed above; and *total altered phenotypes*, cumulative value of all listed above. Digital images of affected embryos and controls were taken at 5 and 8 days post fertilization (dpf) and representative images of eleutheroembryos at 2 days post hatch (dph) (Nikon DXM 1200; NIS-Elements 3.20.01) (Fig S2). Anatomical position used with imaging was as follows: head of the organism directed to the left, the organism in right lateral recumbency with efforts to avoid tilting. In those instances where the alteration could not be visualized, the organism was positioned to increase imaging of the altered feature. After hatching, eleutheroembryos and larvae (10 dph) were sampled for skeletal development, gene expression, and histology (data not shown here).

2.5. Sample preparation for ICP-MS analysis

After 5 consecutive days of embryo collection, bred adults were euthanized via rapid chilling and weighed (to the nearest mg wet weight (ww)). Liver, gonads (ovaries or testes), and skeletal muscle tissue were individually dissected and weighed (mg ww). Skeletal muscle with overlying skin was sampled by removing a rectangular piece of the lateral body wall extending to the caudal margin of the celomic cavity with care to exclude ribs, spinal column, and pectoral fin. To assess Se content in unbred adults, a second, smaller but identical exposure regime was followed for each dietary concentration and treatment group. These tissues along with the 0.1% (w/v) artificial salt water used for the aquaria, 2.5% (w/v) artificial seawater used to hatch *Artemia* cysts, control (commercial diet) and experimental diet, and *Artemia* nauplii were sampled. Samples were processed for quantification of Se content by ICP-MS. Briefly, each tissue and a digestion blank were placed into pre-cleaned, loosely capped glass test tubes and 1 mL of trace metal grade concentrated nitric acid (HNO₃, Sigma–Aldrich) was added to each. Tubes were placed on a heating block at 85 °C overnight or until the fumes turned clear and then removed from the heat and allowed to cool at room temperature for approximately 15 min. Next, digestates were diluted with 9 mL of an acid solution containing 2% HNO₃ and 0.5% hydrochloric acid (HCl, Sigma–Aldrich). All samples were quantified for Se content by inductively coupled plasma-mass spectrometry (ICP-MS; Agilent 7700X ICP-MS equipped with an Octopole Reaction System) in the Pratt School of Engineering at Duke University (Durham, NC) using hydrogen reaction gas at a H₂(g) flow rate of 6 mL min⁻¹ with a high purity standard for trace metals in drinking water (High Purity Standards, Charleston, SC).

2.6. Statistical analyses

Offspring were assessed for abnormal development as proportions of surviving (or hatched, in the case of swim bladder) individuals at 10 dpf using a mixed effects linear model with the sex of the exposed parent(s), dpf, and egg collection date as the explanatory factors. Due its split-plot experimental design, this model included random effects for each trial. This type of model utilizes the median, a more representative value, as these data were not normally distributed and it reduced the influence of outliers. In addition to using the control group as the comparison, a second run of the model was completed using the female only exposure as the comparison group. The Se content of adult tissues was tested for normality using the Shapiro–Wilk test and analyzed using a non-parametric one-way Kruskal–Wallis for significant differences among treatment groups. Differences were considered to be statistically significant at $p \leq 0.05$ (Stata 14, StataCorp LP, College Station, TX, USA).

3. Results

3.1. Se concentrations from ICP-MS

Selenium concentrations in 0.1% and 2.5% (w/v) artificial sea water were 0.02 and 0.17 µg/L, respectively. Addition of *Artemia* nauplii brought the latter to 1.33 µg/L, and the commercial dry food was 4.07 µg/g dw. SeMet spiked food was made by adding 12.5, 25, 50 µg/g dw to commercial diet with final measurements of 11.42, 19.56, and 22.60 µg/g dw, respectively. The detection limit for this ICP-MS method was 0.137 ppb.

Se levels (µg/g ww) in exposed and control fish grouped only by sex showed no significant difference in liver ($p = 0.7145$), gonads (ovaries and testes pooled; $p = 0.968$), or skeletal muscle ($p = 0.330$). When all adults were pooled by only reproductive status (bred or

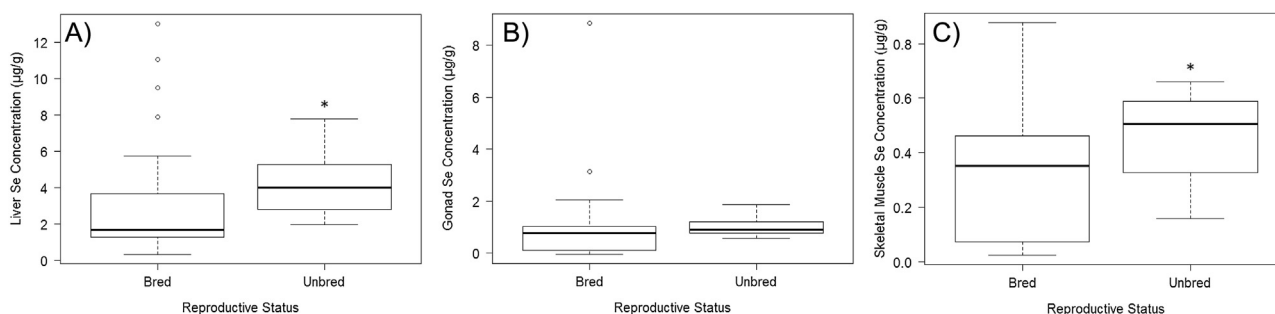


Fig. 1. Boxplots of distributions of Se content ($\mu\text{g/g}$ wet weight) from parental tissue samples dissected from unbred and bred adults: (A) liver, (B) gonads (ovaries and testes were pooled), and (C) skeletal muscle. Black lines represent the medians ($*p \leq 0.05$, non-parametric one-way Kruskal–Wallis).

unbred), breeding caused a reduction in Se in the liver ($p=0.003$) and skeletal muscle ($p=0.032$) compared to unbred fish (Fig. 1). Specifically, in exposed, unbred females, significantly higher Se was found in skeletal muscle ($p=0.045$), but after breeding, liver Se was decreased ($p=0.045$). Also, when exposed males were bred, Se was decreased in skeletal muscle ($p=0.039$). Gonadal Se was not higher in exposed fish than in controls ($p=0.9679$). Ovaries and testes individually were compared among all groups (control, exposed, bred, and unbred); no statistical significance was found.

3.2. Embryo survival, hatchability, and phenotypic alterations

All of the parental dietary SeMet concentrations (nominal – 12.5, 25, and 50 $\mu\text{g/g}$ dw) produced alterations in offspring. Each of these concentrations showed no statistically significant differences from each other with respect to percent total occurrences of phenotypic alterations. However, when offspring of each exposure group was compared to controls, exposure led to significantly higher occurrences of alterations ($p < 0.0001$ for each exposure-control comparison). For this reason, all phenotypic alterations were collapsed into exposed and control embryos grouped by sex of the exposed parent(s), dpf, and collection date (*i.e.*, time from cessation of parental exposure) (Table 2, Fig S1B).

Percent embryo mortality, for all of the treatment groups, increased compared to the control group ($p < 0.0001$), and mortality increased with increasing dpf ($p < 0.0001$) regardless of collection date ($p=0.168$). Percent hatch, of surviving individuals, at 10 dpf was significantly lower in the female only ($p=0.028$) and male only ($p=0.027$) exposure groups, but not the female and male group ($p=0.343$), compared to controls across all collection dates.

Total altered phenotypes proved significantly higher in exposed groups; this was greatest when both parents were exposed ($p < 0.0001$) followed by the female only ($p < 0.0001$) and then male only ($p=0.002$) (Fig. 2A). Individual mixed effects models were run on different categories of alterations (*e.g.*, Pc/Ab edema, craniofacial abnormality, etc., Table 2). Pc/Ab edema proved significantly higher in the female and male group ($p < 0.001$) and the female only ($p < 0.001$) followed by the male only ($p < 0.001$) group, as compared to the control (Fig. 2B, examples in Fig. 3). The female only group was significantly higher than the female and male ($p=0.004$) and male only groups ($p < 0.0001$). Within this category, Pc edema occurred more often in all exposed groups ($p < 0.001$), with the male only group significantly lower than the female only group ($p < 0.0001$). Alteration in spleen color was also higher in all exposed groups ($p < 0.01$). The occurrence of craniofacial deformities was highest in the female and male group ($p < 0.0001$) followed by the female only group ($p < 0.008$) (Fig. 2C, examples in Fig. 4). Within this category, ocular deformities were higher for these groups ($p < 0.001$, 0.033, respectively). In addition, the female and male group had a greater response than the female only

for craniofacial ($p < 0.001$) and ocular deformities ($p < 0.0001$), but the male only group was not lower for either category ($p=0.083$ and $p=0.660$, respectively). Swim bladder noninflation and blood pooling were highest in the female and male group ($p < 0.001$ and $p < 0.0001$, respectively) followed by the female only group ($p=0.027$ and $p < 0.0001$, respectively). Alteration in gall bladder color was observed in the female and male group ($p < 0.001$) and marginally in the male only group ($p=0.077$) compared to controls, with the former higher than the female only group ($p < 0.0001$). Occurrence of notochord malformations were significantly different from the control in the female and male group only ($p < 0.001$), and were significantly higher than the female only group ($p < 0.0001$). In all observation categories except for notochord malformations and swim bladder noninflation, dpf was significant ($p < 0.001$) with more alterations occurring as development progressed. Increasing collection date (*i.e.*, offspring collected further from the termination of parental exposure) was significant for other altered phenotypes and marginally significant for gall bladder color ($p=0.077$) with fewer of these alterations occurring as time from exposure cessation increased.

3.3. Detailed descriptions of selected phenotypic alterations

As stated above, the mixed effects models identified craniofacial abnormalities and edema as major findings, and led us to emphasize these phenotypes in Figs 3 and 4. Edema occurred in groups where mothers had been exposed. *In vivo* morphology of a control eleutheroembryo (Fig. 3A) showed the heart located near the ventral margin with the lipid vesicle slightly caudal and ventral to the intestine. The lipid vesicle proved advantageous in evaluating the degree of edematous change and the association of displaced portions of adjacent structures. The control also showed the spleen as a prominent, dark red body located rostral to the dark swim bladder. The yolk sac is at the ventral caudal portion of the abdominal cavity. Altered offspring contrasted markedly with controls in regard to coloration, location, and shape of these cardiovascular and abdominal structures. Alterations were prominent in the pericardial cavity where clear spaces, consistent with edema, surrounded major vascular structures and pressed against the division between the pericardial (Pc) and abdominal (Ab) cavities (*i.e.*, septum transversum), resulting in slanted (Fig. 3B–C) or vertical dark lines (Fig. 3D) separating these regions of the celomic cavity, and in some cases, undercut the yolk sac (Fig. 3C). The effect of the Pc edema on the morphology of the heart and great vessels was seen in its elongation (tube or stringy heart, Fig. 3B–D). When clear spaces of edema occurred in the abdominal cavity, the dorsal margin of the yolk sac was flattened (Fig. 3B–C) suggesting compression that, when extreme, attenuated vascular structures in- or crossing-the abdominal edematous space (Fig. 3C). Ab edema was typically found in those individuals also exhibiting Pc edema

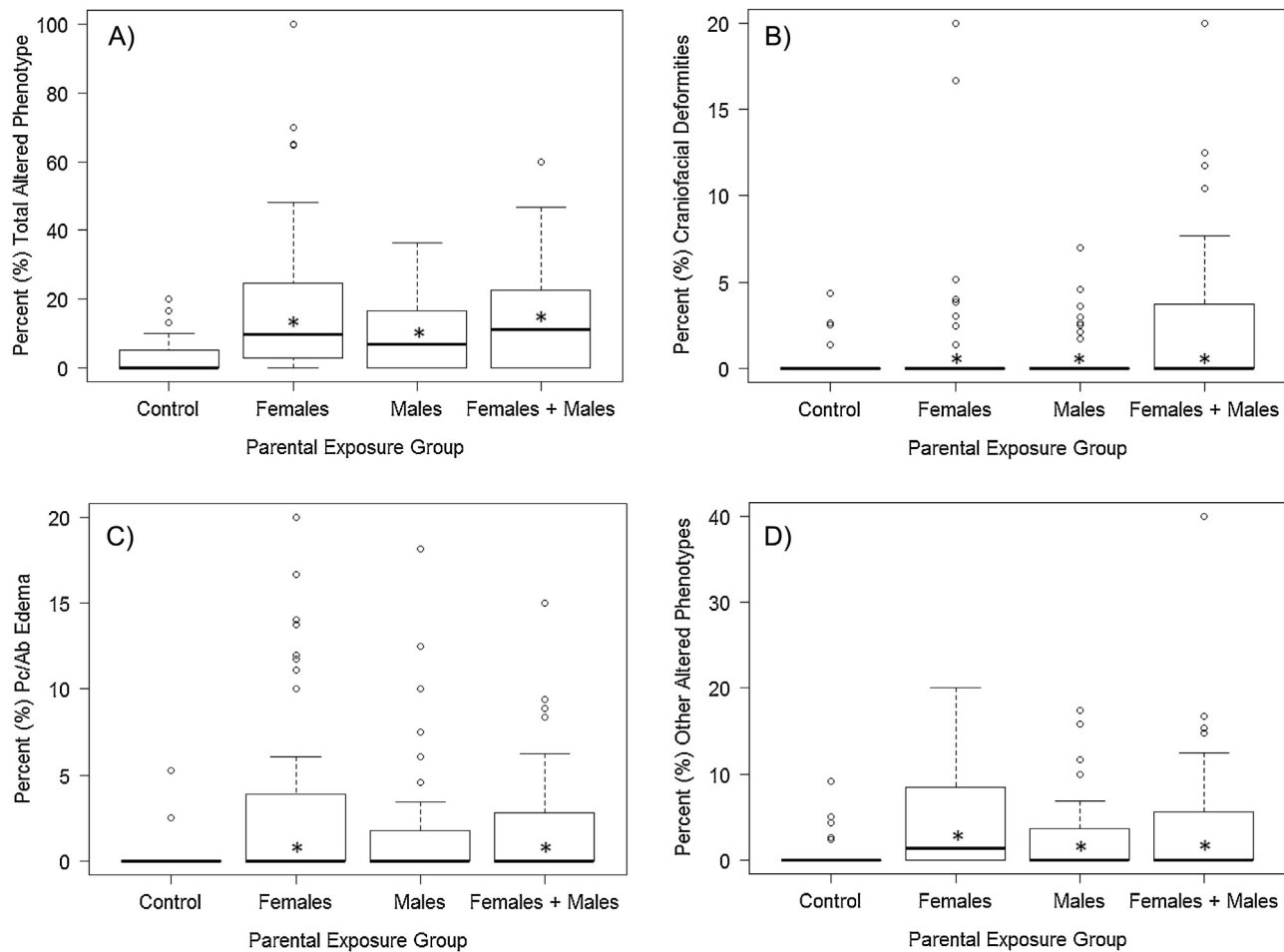


Fig. 2. Percent alterations observed at 10 dpf in the offspring of parental SeMet exposure including (A) total altered phenotypes; (B) craniofacial deformities, (C) Pc/Ab edema, and (D) other altered phenotypes. Percentages were calculated based on the number of surviving individuals at 10 dpf. Black lines represent the medians ($*p < 0.05$).

suggesting the former was an exacerbated state of alteration. Compared to control eleutheroembryos (Fig. 3A), altered individuals, such as that in Fig. 3B, exhibited swelling and bulging of the ventral body wall with the lipid vesicle in close proximity to the yolk sac and occupying a depression in the outer margin of that structure. Blood can be seen in the veins entering the sinus venosus (near the ventral body wall, Fig. 3B) and extending to the elongated atrium followed by the ventricle with its lumen at 90° to the long axis of the attenuated atrium. However, there was little suggestion of blood present in the associated lumens of these structures, perhaps from decreased blood return from either highly attenuated yolk veins in the Ab cavity (Fig. 3C) or edematous tissue surrounding the yolk vein (Fig. 3D). Resultant blood flow into the ventricle and subsequently the aorta appeared greatly diminished (Fig. 3D). We also observed other circulatory alterations, such as blood pooling or aggregation with hemostasis, in both groups where the mother had been exposed. Hemostasis was often associated with Pc edema and pooling of blood in the sinus venosus of the stringy heart, in yolk veins, and/or the caudal vein (Fig. 3B–D). Although not quantified, yolk sac areas following parental exposure were observed to be larger, suggesting less yolk utilization (Fig. 3B–D).

Numerous craniofacial structures were altered and proved significant in offspring where both parents had been exposed (Fig. 4). Eleutheroembryos from control parents showed normal morphology (Fig. 4A, with larger eye field resulting from the highly pigmented portions of contralateral eyes being incompletely superimposed). The appearance of this individual differs greatly from the

representative examples of altered eleutheroembryos in Fig. 4B–D. The eleutheroembryo of an exposed male parent and an unexposed mother (Fig. 4B) shows mandibular extension (prognathism), with the rostral-most portion of the head less developed (maxillary hypoplasia). Ocular deformities varied in their type and severity. The eleutheroembryo of an exposed mother and control father (Fig. 4C), exhibits periocular edema. The eleutheroembryo of an exposure of both parents (Fig. 4D) had an appreciably enlarged mandible, little development of the rostral head and, although not visible from this orientation, only one eye. Also observed were individuals lacking pigmentation in the retina that did not appear to affect orientation and some individuals with partial or complete agenesis of the eyes (not shown).

The presence of other altered phenotypes was higher in all exposed groups as compared to the control ($p < 0.001$, Fig. 2D). Representative phenotypes were imaged *in vivo*, some of which are illustrated in Fig. 5. Fig. 5A, an offspring of a male only exposure group, shows an example of a superficial cellular overgrowth with hemorrhage in the caudal peduncle. Cellular proliferation of the dorso-rostral epithelium was also seen in offspring of female only exposures (Fig. 5C). Caudal alterations were observed including individuals such as the one in Fig. 5B, from a male only exposure group, that had near complete cessation of development of the caudal peduncle. Rare but notable phenotypes were observed including bicephaly, which manifested both as a partial fusion of the eyes near the midline with the formation of a single heart (Fig. 5D) and complete separation that extended past the heart (not

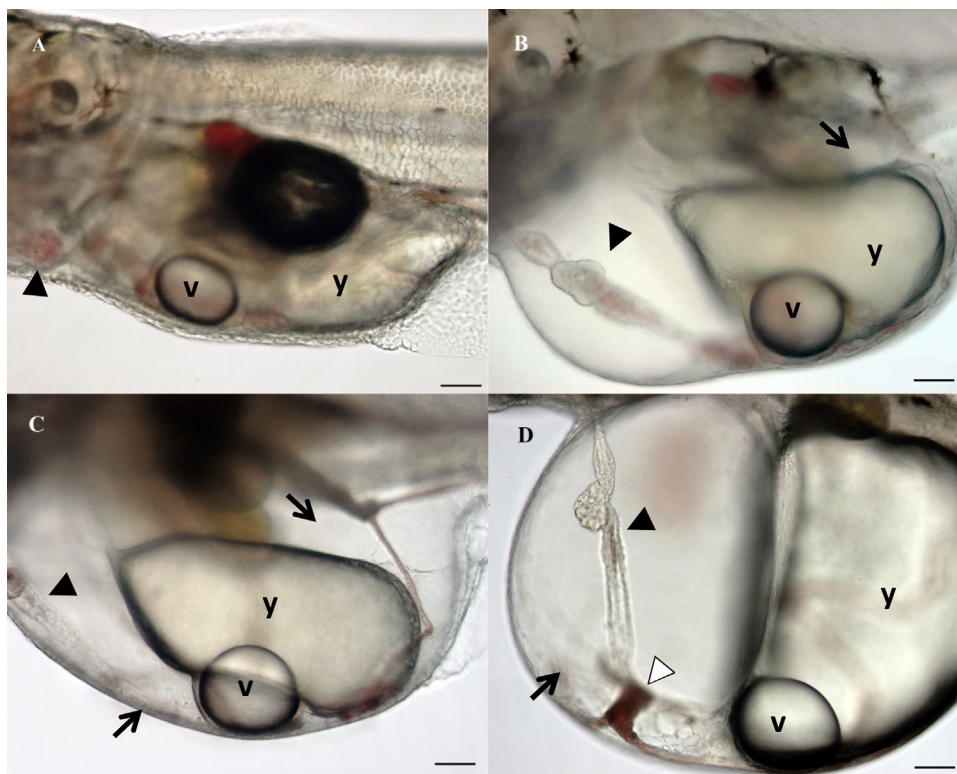


Fig. 3. Examples of pericardial and abdominal (Pc/Ab) edema observed *in vivo* in elutheroembryos 2 dph. Arrows indicate edema, dark arrowheads indicate heart, white arrowheads indicate blood pooling, y denotes yolk, v is the lipid vesicle, and scale bars are 10 μ m. Offspring of (A) control; (B) female only SeMet treatment showing both pericardial and abdominal edema; (C) female only SeMet treatment with both pericardial and abdominal edema; and (D) female and male SeMet treatment exhibiting severe pericardial edema and blood pooling in yolk veins and adjacent sinus venosus.

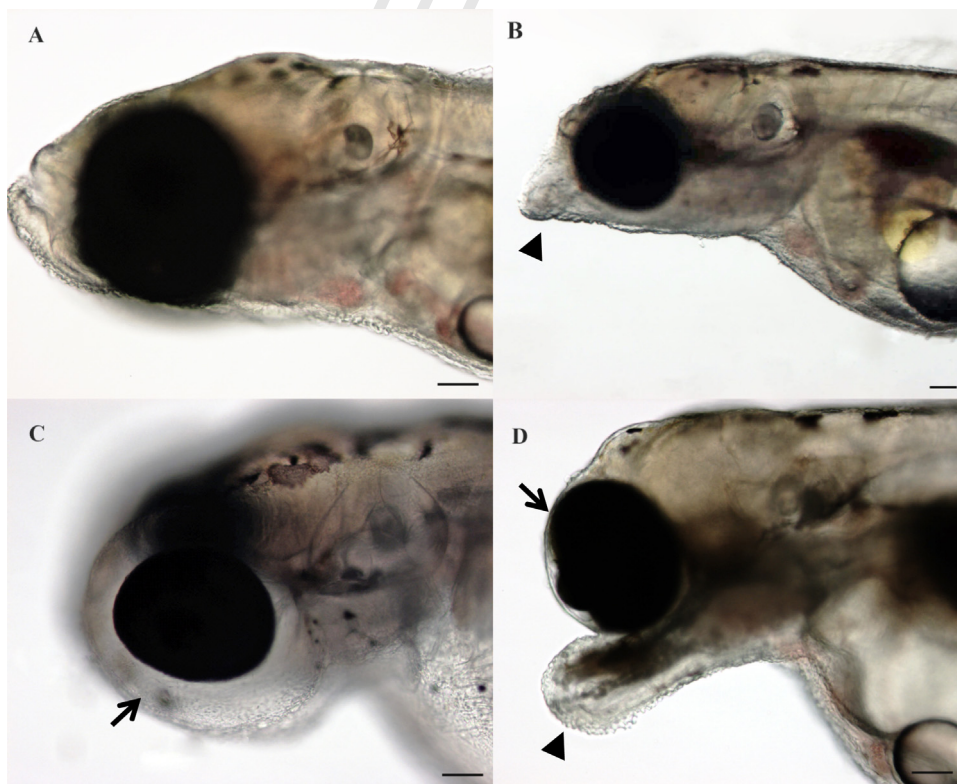


Fig. 4. Examples of craniofacial deformities *in vivo* in elutheroembryos at 2 dph. Arrows indicate ocular deformities, arrowheads indicate mandibular deformities, and scale bars are 10 μ m. Offspring of (A) control; (B) male only SeMet treatment showing mandibular prognathism; (C) female only SeMet treatment with superficial edema of the eye; and (D) female and male SeMet treatment exhibiting cyclopia (there was no contralateral eye), maxillary hypoplasia and mandibular prognathism.

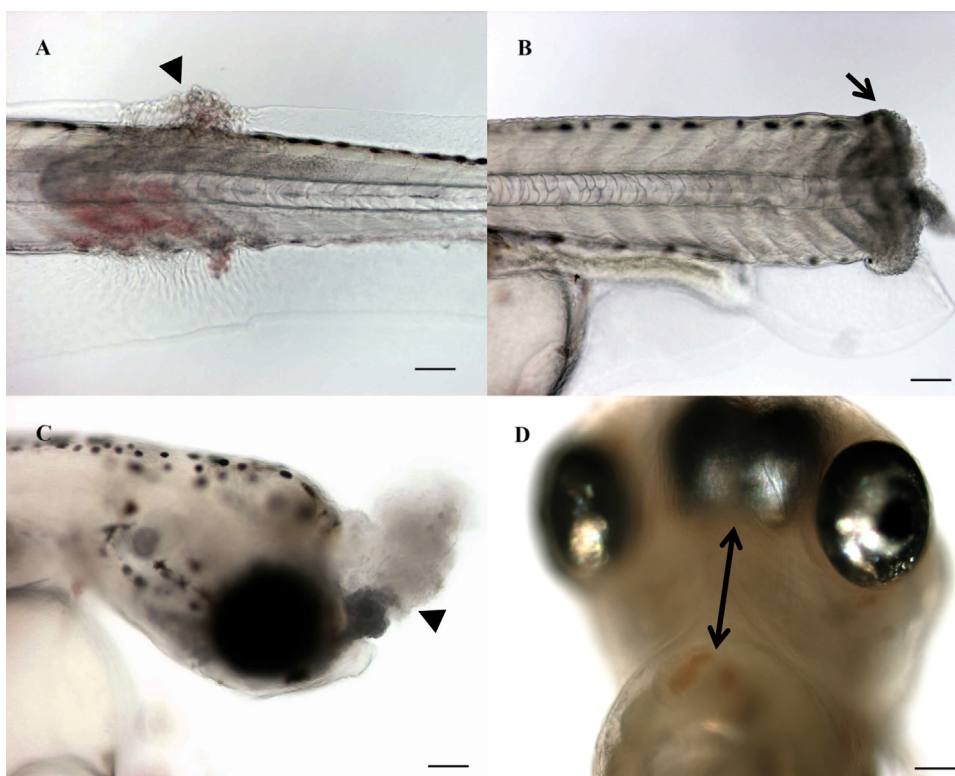


Fig. 5. Examples of *other altered phenotypes* observed in eleutheroembryos 2 dph. Arrow shows truncation of the tail, arrowheads indicate superficial cell masses, and scale bars are 10 μm . Offspring of (A) male only SeMet treatment showing superficial cellular overgrowths with hemorrhage in the caudal peduncle; (B) male only SeMet treatment with truncation of the tail; (C) female only SeMet treatment with dorso-rostral epithelial cellular proliferation; and (D) female and male SeMet treatment exhibiting bicephaly with a shared eye and heart along the midline.

shown). It is important to note that while alterations were analyzed as discrete changes, individual embryos often exhibited multiple phenotypes. Our daily collection of multiple embryos and common observational housing did not allow for serial tracking of individual embryos.

4. Discussion

Parental exposure of medaka to dietary SeMet significantly increased the occurrence of altered phenotypes in their offspring, underscoring the role of diet for each parent, in developmental toxicity. Exposure of both parents was associated with the most alterations in their offspring followed by mothers only then fathers only, compared to controls. In some categories where males only were exposed but failed to result in significant changes to their offspring, the female and male exposure did. Particular categories of phenotypic change appeared to be driven by the sex of the exposed parent.

4.1. Implications of phenotypic alterations in offspring

Pc/Ab edema was significantly higher in all Se- exposed groups when compared to controls, and the results of our model suggest this may be maternally driven. Because Pc- and Ab edema appeared related, we sought evidence of movement of pericardial edema fluid to the abdomen. A passageway, the pericardioperitoneal canal (PPC), is known to occur in primitive fishes and in embryonic mammals (Langman, 1975). In elasmobranchs and white sturgeon (*Acipenser transmontanus*), the PPC remains open to modulate cardiac function via changes in pericardial fluid and transmural pressure (Gregory et al., 2004; Shabetai et al., 1985); its closure dur-

ing development characterizes more derived bony fishes (Gregory et al., 2004). It is possible that reopening of the PPC could account for Ab edema seen in the most extreme cases. Although we did not measure cardiac function or associated survivorship in our study, Hermanutz et al. (1992) reported developmental arrest after hatching of edematous bluegill (*Lepomis macrochirus*) larvae of Se-exposed parents. Furthermore, these larvae of their study failed to absorb the yolk and did not reach the swim-up stage. Aqueous sodium selenite has been shown to induce defects in heart function in zebrafish embryos (Ma et al., 2012), and TCDD embryotoxicity in medaka results in DNA degradation in cells of developing vasculature, particularly the medial yolk vein, as a result of oxidative stress (Cantrell et al., 1996; Thomas and Janz, 2014). Future Se studies should continue to monitor later life stages of exposed individuals to determine significance of these embryonic alterations. While yolk sac edema has been applied to describe changes in embryos exposed to SeMet (Thomas and Janz, 2014), our observations failed to find evidence for edema in the yolk sac. Rather we observed clear spaces in the abdominal cavity that altered the position and shape of this structure, leading us to conclude that, strictly speaking, yolk sac edema was not an accurate term. However, we are in agreement with Thomas and Janz (2014) that Pc- and Ab edema occur after SeMet exposure.

Splenic alteration consisted of a lack of intense red coloration. In fish, this organ serves as a site for hematopoiesis, lymphocyte formation, aged blood cell breakdown, and overall immune response after hatching (see review in Handy et al., 2011; Sorensen et al., 1984). Whether the color change we observed signifies actual alteration in structure and function of the organ is yet to be determined. Examination of adult fish (*Silurus glanis*, *Cyprinus carpio*, *Scardinius erythrophthalmus*) led investigators to regard the spleen

Table 2
Results of the mixed effects linear model for each of the explanatory factors at 10 dpf. The first column lists the explanatory factors and the subsequent columns represent separate effects models compared to the control. Values include coefficient ± SE (top) and p-value (bottom) for each row coinciding to the pre-defined categories used for morphological assessment compared to controls. Grey line separates the exposure groups (above) from the temporal factors (below). Bolded values are $p \leq 0.05$.

Explanatory factors	Mortality	Hatching	Total altered phenotypes	Craniofacial abnormality	Ocular deformities	Pc/Ab Edema	Pericardial edema	Notochord malformation	Spleen color	Gall bladder color	Blood pooling	Swim bladder noninflation	Other altered phenotypes
Female + male exposure	4.06 ± 0.98 – 1.33 ± 1.40	13.52 ± 1.71	2.47 ± 0.41	1.97 ± 0.24	1.22 ± 0.21	1.14 ± 0.20	0.29 ± 0.08	0.28 ± 0.09	0.33 ± 0.07	1.1 ± 0.24	27.18 ± 7.10	3.844 ± 0.41	
Female only exposure	<0.001	0.343	<0.001	<0.001	<0.001	<0.001	<0.001	0.003	<0.001	<0.001	<0.001	<0.001	
Male only exposure	5.65 ± 0.99 – 3.09 ± 1.41	11.40 ± 1.72	1.09 ± 0.41	0.52 ± 0.25	1.81 ± 0.21	1.56 ± 0.20	–0.06 ± 0.08	0.35 ± 0.09	0.09 ± 0.07	1.0 ± 0.24	15.92 ± 7.19	4.16 ± 0.41	
Embryo collection date	<0.001	0.028	<0.001	0.033	<0.001	<0.001	0.442	<0.001	0.175	<0.001	0.027	0.003	
Embryo dpf	5.71 ± 0.98 – 3.10 ± 1.40	5.36 ± 1.72	0.40 ± 0.41	0.28 ± 0.24	0.96 ± 0.21	0.81 ± 0.20	–0.05 ± 0.08	0.30 ± 0.09	0.12 ± 0.7	0.26 ± 0.24	7.33 ± 7.31	<0.001	
	<0.001	0.027	0.002	0.261	<0.001	<0.001	0.521	0.001	0.077	0.276	0.316	0.003	
	1.82 ± 1.32 – 0.48 ± 0.62	–0.84 ± 0.89	0.01 ± 0.16	0.004 ± 0.11	<0.001	–0.07 ± 0.10	–0.05 ± 0.11	–0.08 ± 0.07	–0.07 ± 0.04	–0.21 ± 0.16	2.97 ± 2.77	<0.001	
	0.168	0.441	0.347	0.971	0.482	0.62	0.621	0.206	0.077	0.197	0.283	0.003	
	1.65 ± 0.094	98 ± 0.13	1.89 ± 0.16	0.14 ± 0.02	0.17 ± 0.02	0.13 ± 0.02	–0.01 ± 0.01	0.07 ± 0.01	0.04 ± 0.01	0.12 ± 0.02	3.02 ± 1.81	0.15 ± 0.04	
	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.119	<0.001	<0.001	<0.001	0.095	<0.001	

as an important depot for metals including Se (Jovičić et al., 2015; Yancheva et al., 2014). However, it is difficult to assess Se content in the developing fish spleen. To fully evaluate embryo/larval spleen function, examinations need to be extended to the tissue and cellular level and continued temporally.

Craniofacial abnormalities proved significant in offspring where both parents had been exposed, and the results of our model suggest a paternal influence for this phenotype. Similar jaw deformities have been observed in red shiners (*Notropis lutrensis*) (Lemly, 1993), bluegill (Lemly, 2014), and northern pike (*Esox lucius*) (Muscatello et al., 2006) and, while not immediately lethal, affect closing of the mouth and potentially could affect feeding and respiration (McKim, 1977; McKim et al., 1976). Within the craniofacial abnormalities category, the presence of ocular deformities exhibited the same statistical pattern among treatment groups. Sections of eyes of zebrafish embryos exposed to flame retardant compounds revealed alterations of pigment in the retina due to enhanced apoptosis (Dong et al., 2014). In our future studies, we will specifically address retinal apoptosis to determine factors that could lead to this phenotype. The function of selenoproteins and the antioxidant properties of excess Se in postnatal mammalian ocular physiology are known to cause oxidative damage via redox cycling and result in cataracts (Flohe, 2005). *In situ* hybridization (ISH) has been used to localize specific selenoprotein expression in retina of developing zebrafish (Thisse et al., 2003), and confocal X-ray fluorescence imaging of zebrafish offspring of dietary SeMet exposed mothers showed accumulation of Se in the eyes of hatchlings (Choudhury et al., 2015). The highest concentrations appeared in the lens-core followed by the lens-epithelium, and these investigators regarded sulfur-substitution as the mechanism in lens proteins, as opposed to the oft-studied oxidative stress mechanism, and suggested that the eye may be an ideal site to test mechanisms of toxicity (Choudhury et al., 2015). Taken together, even minor ocular impairments in fish could be expected to have direct effects on behavior and survival including visual attraction to food, migration within the water column, and detection of predators (Guthrie, 1986). The finding of specific craniofacial abnormalities suggest that the exposure of parents affords the opportunity for Se to interfere with early processes in development, such as upstream regulatory genes (Choy and Cheng, 2012; Gunhaga et al., 2003; Wang et al., 2005).

Absent or partially inflated swim bladders were significantly higher in both treatments where the mother was exposed. To achieve swim up behavior, eleutheroembryos must first inflate their swim bladders and subsequently decrease body density to attain neutral buoyancy (Lindsey et al., 2010). Because the swim bladder of the medaka is a single chamber as compared to the two chambered bladder in zebrafish, spinal deformation associated with the latter species may not occur or may manifest differently (Marty et al., 1995). The medaka swim bladder inflates minutes after hatching via the pneumatic duct, an outgrowth of the foregut endoderm which is innervated by the peripheral nervous system, and the production of surfactants that reduce surface tension at the air-liquid interphase of this organ (González-Doncel et al., 2003). Reduced inflation, extending in some individuals to noninflation of the swim bladder, means that affected larvae must constantly move increasing oxygen consumption and energy expenditure as they attempt to maintain position in the water column, and such constant motion of the sinking medaka leads to extreme emaciation (Marty et al., 1995).

An unexpected result was the response when both parents were exposed. In more than half of the pre-defined categories, this exposure group showed the highest occurrences of alterations compared to controls. In some categories, this group proved higher than the female only group, and interestingly, higher even in those categories where the male only treatment showed no significant differences. Also unforeseen was the response in the male only

exposure group where gall bladder color, was of particular interest. As with the spleen, change or absence of color was used as an indicator for organ dysfunction; either the gall bladder itself or its contained biliary products of the liver may be responsible for such change. Also, since the gall bladder concentrates bile via removal of water, this may be associated with color change (Volz et al., 2008) and is important avenue for further research.

While our analysis addressed specific developmental alterations as distinct entities, it is important to note that many individual offspring exhibited multiple malformations. Many of these severely deformed embryos failed to survive the hatching process. In our study, embryo mortality increased with increasing dpf and more time for development resulted in more sites of alteration. SeMet is apparently affecting components that are needed in multiple host sites for normal development and the alterations of specific proteins and genes could account for such widespread change.

4.2. Selenium in adult tissues

Adult tissue Se concentrations in our study varied among tissue types after acute exposure. This is similar to other studies of short-term dietary Se exposure. A 2 week dietary SeMet exposure to juvenile rainbow trout (*Oncorhynchus mykiss*) was followed by an analysis of tissue-specific distribution and speciation of Se (Misra et al., 2012). They reported selenocysteine in all tissues except liver. Highest SeMet concentration occurred in gill, and only traces of selenite, restricted to skeletal muscle were found (Misra et al., 2012). *In situ* caged (21 days), wild lake chub (*Couesius plumbeus*) in one reference and three northern Canadian study lakes resulted in Se body burdens that were higher in Se-contaminated lakes relative to fish from reference site, with SeMet being the predominant form (Phibbs et al., 2011). In cutthroat trout (*Oncorhynchus clarki lewisi*), a high correlation and significance between Se residues in liver, muscle, and gonad tissue and Se concentrations in eggs were seen in females from rivers downstream of surface coal mines (Kennedy et al., 2000; Rudolph et al., 2008). Muscatello et al. (2006) made a linkage between Se concentration in eggs and resultant change in offspring (*i.e.*, edema skeletal, craniofacial, and fin deformities) with increased Se concentrations in muscle, liver, and bone in female northern pike. Interestingly, laboratory experiments in which parents were exposed and changes in offspring detected utilizing asynchronous aquarium model fish may highlight differences between breeding strategies compared to the above synchronous species. With each of the aquarium model fish, the time required for gametogenesis is shorter than that required for synchronous breeders, an aspect that may reflect in the differences between body burdens of Se required to produce offspring abnormalities. We detected a reduction in specific adult tissue Se concentrations following breeding, suggesting a utilization or transfer of this excess Se to gametes during vitellogenesis and/or spermatogenesis.

Our results show a reduction in liver and skeletal muscle tissue Se with breeding. The decrease in liver Se is likely driven by exposed females, due to the hepatic role in vitellogenin synthesis (Shibata et al., 2011). Interestingly, the decrease in skeletal muscle Se proved prominent in unbred, exposed fish of both sexes. In higher vertebrates, ingested SeMet is absorbed from the small intestine, enters the blood stream and is incorporated into organs with high rates of protein synthesis, *viz.* liver, erythrocytes, and skeletal muscle (Schrauzer, 2000). No such reduction was observed in either the pooled or individual ovaries or testes. The short exposure time may not have allowed for significant amounts of Se to accumulate in these tissues as gonads redistribute Se and accumulate it later than other organs (Kleinow and Brooks, 1986). Importantly, Thomas and Janz (2015) specifically addressed Se in mature eggs of zebrafish, an asynchronous species, and reported Se concentra-

tions higher than that of the ovary. In medaka, Se may be shuttled directly from the diet to the eggs, making transient shifts in dietary Se important (Conley et al., 2014). The low ovarian Se values that we observed may be a result of the asynchronous reproductive strategy of medaka, the acute exposure duration and/or the redistribution of Se by this organ, and our future work will specifically examine Se concentration in mature eggs.

A general paucity of direct consideration of males is seen in reviews of Se and fish studies. Previous studies have varied in their sampling of and/or reports on male tissue analysis. Our review indicated a near absence of consideration of Se content of testis and milt, particularly in field-based studies. Even in studies where testes were sampled, emphasis remained on the female contribution. After Hamilton and Waddell (1994) reported the concentration of Se in milt of wild razorback suckers (*Xyrauchen texanus*) from Se-laden waters of the middle Green River, CO to be within the range of reference fish, they subsequently focused attention on eggs only. Ogle and Knight (1989) conducted a dietary exposure with fathead minnows, sampling whole body and gonadal tissues for Se content. At the highest concentration (30 ppm), they reported a Se content of testis that was significantly lower than that of ovarian tissue; but, importantly, this value was significantly higher than the reported corresponding male control. These authors focused the remainder of their report on the vitellogenic female with no further attention given to the male role. Hermanutz et al. (1992) examined aqueous selenite exposure in bluegills. After 258 days of exposure, increased Se in both ovaries and testes was observed, and testicular Se levels became the highest of all tissues by 356 days of exposure. As with the above study, discussion in the Hermanutz et al. report focused on liver and ovaries as accepted routes of toxicity and provided no further mention of testis. Baumann and Gillespie (1986) compared bluegill and largemouth bass (*Micropterus salmoides*) tissues from a reference lake to coal-ash affected impoundments and found higher Se levels in ovaries than in testes for both species. Individually, whole body and testis Se concentrations appeared higher in exposed males compared to reference males. When examining testis level as a percentage of whole body levels, testis concentrated Se at lower body burdens, then plateaued as carcass Se concentration increased, finally being surpassed by whole body levels with increasing Se exposure concentration. However, that study did not test resultant embryos so it is unknown if these body burdens affected sperm and related embryo quality.

While we strongly agree with the importance and validity of maternal transfer studies, we believe attention should also be given to sperm quality. Almost no Se exposure studies have investigated the testis as a site of Se toxicity. Weber et al. (2008) reported delayed testicular maturation in fathead minnows collected from Junction Creek, Sudbury, Ontario. Areal analyses of tissue sections of spermatogonia, spermatocytes, and mature sperm showed the quality of cells involved in spermatogenesis to be diminished. As support for this, they reported apoptotic sperm (Weber et al., 2008). Because this was the sole report we found centered on testicular alterations, there is a need for future studies to assess testicular morphology and sperm maturation. One study from our laboratory indicated that such an approach can be taken in medaka to evaluate gene expression and testicular morphology (Miller et al., 2012). The links between Se and male reproductive performance have been well studied in mammals, particularly mice and livestock, linking Se-deficient and Se-excess diets with semen quality and fertility via selenoproteins and oxidative damage mechanisms (Ahsan et al., 2014). However, these mechanisms are less well known in teleost fish and our results suggest that additional studies of testis should include selenoprotein expression and antioxidant system alterations. Duration of short-term exposures overlapped with timing required for spermatogenesis

(Egami and Hyodo-Taguchi, 1967; Saiki et al., 1997). Grier (1976) investigated the fine structure of testicular cells in medaka. Cells lining testis cysts were shown to ingest residual bodies cast of by spermatids. Such cells would be of interest to see their response to toxic and/or apoptotic alterations (Grier, 1976). With respect to germ cell mutagenesis and resultant genomic instability, medaka have played a major role as a model for human reactions to cosmic ray nuclei (Shimada et al., 2005) and in studies of chemical mutagens (Shima and Shimada, 2001). Winn et al. (2008) evaluated offspring mutations transmitted from ethylnitrosourea (ENU) exposed male transgenic λ medaka. Use of the insertion of a target gene in this line provided a way to assess stage-specific germline mutagenesis and implicated these changes in postfertilization genomic instability in offspring. A detailed approach such as this should be taken in evaluating possible genetic and epigenetic effects in germ cells and determining whether some of the male specific effects might illuminate selenium investigations.

5. Conclusions

In summary, the results of our study have shown that the offspring of Se exposed parents have significantly more altered phenotypes, often multiple changes within a single individual, when compared to unexposed fish. This response was greatest in those treatment groups that exposed both parents, even when the male only exposure groups were not higher than controls. Importantly, particular types of phenotypic alteration appear to be driven by the sex of the exposed parent. These findings, together with the rarity of studies testing male contributions in environments with excess Se, suggests that additional attention be given to effects on males. Medaka proved a beneficial model organism to demonstrate how short-term Se exposure can substantially affect an asynchronous breeder, targeting vitellogenesis, oocyte maturation, and possibly spermatogenesis. The level of detail we have provided in our morphologic assessment early life stage medaka will facilitate the linkage of phenotype to genotype and better inform the longer term consequences of Se exposure. Future work will focus on other routes of exposure as well as on the differences in toxicity between selenium species. Additionally, we suggest the use of other asynchronous fish models in conjunction with species of interest to link mechanism to environmental relevance.

Uncited references

Finley and Garrett (2007), Lee et al. (2010), Lemly (1982) and Palace et al. (2004).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.aquatox.2015.11.004>.

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