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Zebrafish (*Danio rerio*) vary by strain and sex in their behavioral and transcriptional responses to selenium supplementation

Maia J. Benner^a, Robert E. Drew^{a,1}, Ronald W. Hardy^b, and Barrie D. Robison^{c,*}

Maia J. Benner: maiabenner@vandals.uidaho.edu; Robert E. Drew: rdrew@uidaho.edu; Ronald W. Hardy: rhardy@uidaho.edu

^aDepartment of Biological Sciences, University of Idaho, Moscow, ID, 83844-3051, USA

^bAquaculture Research Institute, Hagerman Fish Culture Experiment Station, University of Idaho, 3059 National Fish Hatchery Road # F, Hagerman, ID 83332-5851, USA

^cDepartment of Biological Sciences, University of Idaho, Initiative for Bioinformatics in Evolutionary Studies, University of Idaho, Moscow, ID, 83844-3051, USA

Abstract

We used the Nadia, Gaighatta, Scientific Hatcheries, and TM1 zebrafish (*Danio rerio*) strains to test the hypothesis that variation among populations influences the behavioral and transcriptional responses to selenium supplementation. When fed a diet with control levels of selenium, zebrafish strains differed significantly in behavior, characterized as their mean horizontal and vertical swimming positions within the tank. The four strains also differed in brain expression of selenoprotein P1a (*sepp1a*), glutathione peroxidase 3 (*gpx3*), thioredoxin reductase 1 (*txnr1*), and tRNA selenocysteine associated protein 1 (*secp43*). Iodothyronine deiodinase 2 (*dio2*) did not differ among strains but showed a sex-specific expression pattern. When supplemented with selenium, all strains spent a greater proportion of time near the front of the tank, but the response of vertical swimming depth varied by strain. Selenium supplementation also caused changes in selenoprotein expression in the brain that varied by strain for *sepp1a*, *secp43*, and *dio2*, and varied by strain and sex for *txnr1*. Expression of *gpx3* was unaffected by selenium. Our data indicate that selenium homeostasis in the brain may be a regulator of behavior in zebrafish, and the strain-specific effects of selenium supplementation suggest that genetic heterogeneity among populations can influence the results of selenium supplementation studies.

Keywords

Selenium; Selenoproteins; Zebrafish; Gene expression; Behavior; Genotype by environment interaction

1. Introduction

Selenium (Se) is an essential trace element for maintaining normal brain function in humans and other vertebrates (Hawkes and Hornbostel, 1996; Rayman, 2000; Richardson, 2005). The brain is a privileged tissue in terms of Se supply, experiencing only slightly reduced Se levels during dietary Se deficiency and receiving priority uptake as compared to other

tissues during re-supplementation (Schweizer et al., 2004; Barbazuk et al., 2007). Se homeostasis in the brain is maintained primarily by selenoprotein P, a member of a class of Se-dependent proteins known as selenoproteins (Hill et al., 2004; Schweizer et al., 2004; Richardson, 2005; Nakayama et al., 2007). Selenoproteins produce most of the biological effects of Se (Burk et al., 2003; Suzuki, 2005) and decreases in Se status can reduce their expression (Dumitrescu et al., 2005; Hesketh, 2008). If Se intake is suboptimal, selenoprotein function may be impaired, resulting in altered Se metabolism and neurological function.

The changes in neurological function and behavior associated with variation in Se status have been studied primarily in rodents. Selenoprotein P gene (*sepp*) knockout mice fed a low Se diet exhibited severely altered motor function, which could be prevented with elevated dietary Se (Hill et al., 2003, 2004; Schweizer et al., 2004). Altered spatial learning and memory as well as disrupted hippocampus synaptic plasticity was also observed in *sepp* knockout mice fed 1ppmSe, while a similar phenotype was seen in the control mice fed a Se-deficient diet (0ppmSe) (Johnsson et al., 1996). Similar, but less severe, neurological dysfunction has also been observed in non-transgenic mice fed Se-deficient diets. After 15 days on a Se-deficient (~0.01ppmSe) diet, Castaño et al. (1997) observed altered neurotransmitter turnover in the prefrontal cortex of rats. Watanabe and Satoh (1995) also observed that mice bred from nutritionally Se-deficient (<0.02 ppm Se) dams and maintained on the same Se-deficient diets after weaning were less active, defecated more, entered the center of an open field less frequently, and had impaired performance in Morris water maze tests. Conversely, high-dose Se supplementation (1ppmSe) for 6 months in mice resulted in increased open field activity and decreased sniffing behavior, indicating a heightened arousal state (Boylan et al., 1990).

In humans, several neurological conditions have also been associated with changes in Se status. Low blood Se levels have been linked to intractable seizures in children (Schweizer et al., 2004), while supplementation with Se has been shown to mitigate them (Rayman et al., 2006). Some evidence also suggests that insufficient Se levels are associated with Parkinson's and Alzheimer's disease (Schweizer et al., 2004). Se deficiency is also associated with mood disorders such as depression and anxiety, and increased incidences of confusion and hostility (Rayman, 2000). There is controversy as to whether Se supplementation can ameliorate these mood and anxiety disorders. Some studies have shown that Se supplementation affects mood (Finley and Penland, 1998; Benton, 2002; Shor-Posner et al., 2003), while others detected no such relationship (Hawkes and Hornbostel, 1996; Rayman et al., 2006). The lack of concordance among human studies regarding Se supplementation and mood may result from genetic variation among populations in the biochemical pathways that process Se (Hesketh, 2004, 2008; Méplan et al., 2006, 2007, 2008).

If genetic variants occur in selenoprotein gene regions that are involved in Se incorporation, the synthesis and activity of selenoproteins may be affected, altering individual Se requirements (Méplan et al., 2006; Hesketh, 2008). Méplan et al. (2007) observed two single nucleotide polymorphisms (SNPs) in human *sepp* that influenced individual response to Se supplementation through complex interactions with sex and body mass index. The SNPs in *sepp* were also associated with variation in activities and concentrations of other selenoproteins, specifically glutathione peroxidase 1, 3, and 4 and thioredoxin reductase 1. Additionally, the identification of several SNPs in the selenoenzymes involved in the synthesis of selenoproteins, suggests that genetic variation in these selenoenzymes may also affect inter-individual variation in Se metabolism, and possibly minimum dietary Se requirements (Dumitrescu et al., 2005; Méplan et al., 2006; Hesketh, 2008). While it is clear that genetic variation can influence the physiological response to Se, no studies have yet

investigated whether variation in selenoprotein expression among populations or individuals can affect behavioral and transcriptional responses to Se supplementation.

Here, we use the zebrafish (*Danio rerio*) to test the hypothesis that variation among study populations can influence the behavioral and transcriptional responses to Se supplementation. Recent work has established the zebrafish as an excellent neurobehavioral model (Gerlai, 2003; Guo, 2004; Egan et al., 2009). For example, anxiety and stress responses in zebrafish change when exposed to environmental and pharmacological manipulations such as caffeine and fluoxetine (an antidepressant) in a similar pattern to humans and rodents (Egan et al., 2009). Zebrafish are also a potentially useful model for Se supplementation studies, as their selenoproteins have been characterized (Kryukov and Gladyshev, 2000). In addition, zebrafish appear to utilize selenocysteine (Sec), the amino-acid form of Se, to a greater extent than other eukaryotes (Kryukov and Gladyshev, 2000). Zebrafish also have multiple copies of selenoprotein genes, novel selenoproteins, and zebrafish *sepp* contains the greatest number of Sec residues across all species studied to date. Finally, behaviorally distinct and genetically discrete strains of zebrafish (Robison and Rowland, 2005; Moretz et al., 2007a,b; Oswald and Robison, 2008) also allow us to test the effects of Se supplementation in multiple populations. In this paper, we test the following predictions of our hypothesis: 1) Se supplementation elicits a behavioral response in zebrafish, 2) Se supplementation elicits transcriptional changes of selenoprotein genes in the brain, and 3) Populations that vary in basal levels of selenoprotein expression in the brain will vary in behavioral and transcriptional responses to Se supplementation.

2. Materials and methods

2.1. Zebrafish strains and husbandry

We tested the effect of Se supplementation in four strains of zebrafish (*D. rerio*, Cyprinidae). Of the four strains, two strains were bred in the laboratory for more than 30 generations (Scientific Hatcheries and TM1) and two strains were more recently derived from wild populations in India (Nadia and Gaighatta). The particular strains were selected because a previous microarray experiment (Robison et al. unpublished) from our laboratory indicated that they vary in expression of selenoprotein P1a (*sepp1a*) in the brain. All fish were one year old, sexually mature adults at the time of the experiment. Prior to the experiment, strains were bred and reared in separate aquaria and in the same recirculating system (Aquaneering, San Diego, CA USA) so that they experienced similar environmental conditions. Fish were maintained at 28.5 °C, on a constant 14 h light:10 h dark photoperiod. A total of 120 fish were used in the study (n=30, per strain) with ten fish per strain assigned to each dietary treatment (see Section 2.2). While we attempted to use an approximately equal number of males and females, this was not possible for the TM1 strain, in which we observed a highly male-biased sex ratio (29 males and 1 female).

At the start of the experiment, each fish was randomly assigned to a separate 1-L aquarium (width: 5 cm, height: 15 cm, depth: 25 cm) that was part of the re-circulating system in which they were reared. To minimize social transmission of behavior during the experiment, fish were visually isolated from one another by the placement of a paper barrier between each aquarium. Aquaria were marked with a numeric identifier so that information regarding strain and diet were unknown to the observer throughout the collection of behavioral data. The fish were allowed to acclimate to their new aquaria for two months. During this time, they were fed a combination of commercial flake food (Tetra, Melle, Germany) and *Artemia* nauplii (INVE Aquaculture, Salt Lake City, UT, USA) three times daily. Procedures involving animals were approved by the University of Idaho Institutional Animal Care and Use Committee.

2.2. Experimental diets

Immediately following the acclimation period, the fish were fed one of three soy protein concentrate-based pellet food diets differing only in the level and form of Se: control (1.4 ppm Se), selenomethionine supplementation (7.1 ppm Se), and sodium selenite supplementation (5.6 ppm Se). These diets were produced at the University of Idaho Hagerman Fish Culture Experiment Station (Hagerman, ID, USA). Fish meal and other dietary ingredients provided the Se in the control diet, no additional Se was added. The Se supplemented diets were supplemented directly with either an organic (selenomethionine, Sigma-Aldrich Corp., St. Louis, MO, USA) or inorganic (sodium selenite, Sigma-Aldrich) Se. Total levels of Se in each diet were assayed by the Analytical Sciences Laboratory of the University of Idaho.

Ten fish from each strain were randomly assigned to each of the three diets. Fish were fed the experimental diets three times daily (8 ± 2 mg/feeding) for a total of seven weeks. During the final five weeks of the dietary treatments, behavioral data were collected as described in Section 2.3.

2.3. Behavioral assays

Behavior data were collected at three observation times daily (0900, 1200, and 1500) immediately prior to being fed. Each tank was delineated into six 2-cm vertical zones. A scan sampling method was used to note the vertical and horizontal locations of each fish in response to an observer. Scan sampling is a behavioral observation method used to assess the proportion of time an individual spends in a specific behavioral state, such as time spent in vertical or horizontal zones (Altmann, 1974). At each daily observation time, the location of the fish within their individual tank was recorded each second for a period of 3 s. We performed this scan sampling 3 times per day for a total of 9 scans per day, and the daily average of these measurements was used to determine the proportion of time each fish spends in a certain location within their tank. Vertical location was coded with an integer (1 through 6, where 1=surface, 6=bottom). Horizontal location was recorded as a binary variable, with 1 indicating the fish was within one body length of the front of the tank nearest to the observer, and 0 indicating the fish was not. These data were then used to calculate two indices of behavior. "Vertical Depth" was the daily mean vertical position for each individual while "Horizontal Position" was the daily mean horizontal score, equivalent to the proportion of time the animal was within one body length of the front of the tank nearest the observer. Behaviors were scored by the same observer from a distance of 0.5 m each weekday for a period of five weeks. In total, we collected 25 repeated measures (daily means) of each behavior for each fish. These behaviors are known to vary with time spent in captivity and are related to stress, anxiety, and predator avoidance in fishes (Reinhardt, 2001; Robison and Rowland, 2005; Egan et al., 2009).

2.4. Tissue collection

At the end of the seven week supplementation period, the fish were anesthetized with 170 mg L⁻¹ MS222 (Tricaine methanesulfonate, Argent Laboratories, Redmond, WA, USA) and euthanized by decapitation. Whole brain samples were then removed from each fish, immediately homogenized in TRIzol (Invitrogen, Carlsbad, CA, USA), and total RNA extracted following the manufacturer's protocol.

2.5. Analysis of gene expression

We analyzed the expression of four selenoprotein genes and one Sec-associated protein gene in the brain: selenoprotein P1a (*sepp1a*), glutathione peroxidase 3 (*gpx3*), iodothyronine deiodinase 2 (*dio2*), thioredoxin reductase 1 (*txnr1*), and tRNA Sec-associated protein 1

(*secp43*) (see Table 1 for primer sequences). We focused on *sepp1a* because of its importance in brain Se homeostasis and because previous microarray analyses in our laboratory showed that expression of *sepp1a* in the brain varied among the four zebrafish strains used in this study (Robison et al. unpublished). We also sought to compare expression responses of *sepp1a* to Se supplementation with the responses of other selenoproteins. We therefore measured expression of *gpx3*, *dio2*, and *txnrd1*; each was chosen as a representative from the prominent Se-dependent protein families: the glutathione peroxidases, thioredoxin reductases, and deiodinases (Birringer et al., 2002). We also measured expression of *secp43* as a representative from the Sec incorporation machinery proteins (Méplan et al., 2006). We chose these specific members of the selenoprotein gene families because previous microarray data from our laboratory indicated that they were expressed in the zebrafish brain (microarray data found at NCBI Gene Expression Omnibus (GEO) under accession number GSE11107 (Drew et al., 2008)). Expression of each gene was measured using SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), on an ABI 7900HT Real-Time PCR System as described in Drew et al. (2008). Eukaryotic elongation factor 1 α (*eef1a*) was used as a reference gene for quantifying relative expression; expression of *eef1a* (raw cycle thresholds, C_T) was unaffected by Se supplementation.

2.6. Statistical analysis

The effect of dietary Se supplementation and strain on behavior was tested using a mixed model ANOVA with repeated measures, using PROC MIXED within the statistical program SAS version 9.1 (SAS Institute, Cary, NC, USA). Daily means for Vertical Depth and Horizontal Position (n=25 days) were analyzed as the repeated measure with individual as the experimental unit. Because daily behavioral means were temporally related and did not satisfy the assumption of independence, we used an autoregressive correlation structure in our repeated measures design. The effect of Day refers to the time over the 25 day period, not the daily time of observation, and did not show an obvious pattern over time. The main model consisted of a fully crossed factorial design of Strain, Diet, Sex, and Day of measurement. Model residuals were tested for normality, and in the case of Vertical Depth no violations were detected. Residuals from the analysis of Horizontal Position were significantly non-normal but this was rectified using a square root transformation.

For the analysis of gene expression, we employed a mixed model ANCOVA to test the effect of Strain, Diet, and Sex on mRNA levels of each selenoprotein gene (C_T). Individual fish were the experimental units, while duplicate measurements of gene expression were treated as repeated measures. Plate was included in the model as a blocking factor. Rather than normalizing expression of the target genes before analysis, the expression level (C_T) of the reference gene, *eef1a*, was used as a covariate in an approach similar to Yuan et al. (2006). We have used this technique successfully in other studies of zebrafish gene expression (Drew et al., 2008; Robison et al., 2008). Normality of model residuals was examined and no violations were detected.

Due to the male-biased sex ratio of the TM1 strain, a different statistical model was required for the analysis of two genes with sexually dimorphic expression patterns (*dio2* and *txnrd1*). In the case of *dio2*, we removed the data for the TM1 strain because expression of this gene was sexually dimorphic and there was only one female in the TM1 data set. We also observed a complex interaction between Sex, Strain, and Diet on brain expression of *txnrd1*; therefore tests of the effects of Diet and Strain were performed within each sex for this gene and the TM1 strain was removed from the analysis.

3. Results

3.1. Effect of strain and Se supplementation on behavior

We observed a significant effect of Strain ($F_{3, 105} = 58.00$, $P < 0.0001$) and a Strain by Diet interaction ($F_{6, 105} = 7.76$, $P < 0.0001$) on Vertical Depth. When fed the control diet, the Gaighatta (G) and TM1 (T) strains occupied a significantly lower position in the water column than the Nadia (N) and Scientific Hatcheries (S). When fed the Se supplemented diets, however, the G and N strains occupied a significantly lower position in the water column relative to fish fed the control diet, while the S and T strains swam significantly higher (Fig. 1). The significant interaction between Strain and Diet indicates that strain influenced how Se supplementation affected Vertical Depth. The response of Vertical Depth was also specific to the chemical form of Se; each strain differed in the form of Se that influenced their position in the water column. We also observed a significant effect of Day on Vertical Depth ($F_{23, 2438} = 2.61$, $P < 0.0001$), and therefore report least square treatment means, adjusted for the effect of Day, from the mixed model repeated measures ANOVA. In addition, we observed a small but significant effect of Sex on Vertical Depth ($F_{1, 105} = 5.77$, $P = 0.0181$), with males (mean = 4.6 cm) occupying a significantly lower point in the water column than females (mean = 4.2 cm). P-values for all terms in the statistical model of the behavioral data are reported in Table 2.

We observed a significant effect of Strain (Fig. 2a; $F_{3, 105} = 31.18$, $P < 0.0001$) and Diet (Fig. 2b; $F_{2, 105} = 5.20$, $P = 0.0070$) on Horizontal Position. Fish from the G strain had significantly lower Horizontal Position (i.e. spent significantly less time within one body length of the observer) than fish from the N strain, and both G and N fish had significantly lower Horizontal Position than the S and T strains (Fig. 2a). Se supplementation caused a small but significant increase in Horizontal Position in all strains; the mean Horizontal Position of fish fed the control diet was significantly lower than in fish fed the selenomethionine and sodium selenite supplemented diets (Fig. 2b). We also observed a significant effect of Day ($F_{23, 2438} = 6.05$, $P < 0.0001$) and a Strain by Day interaction ($F_{69, 2438} = 1.33$, $P = 0.0362$) on Horizontal Position, and therefore report least square treatment means, adjusted for the effect of Day, from the mixed model repeated measures ANOVA. Similar to our observations with Vertical Depth, the effect of Day on Horizontal Position did not show an obvious pattern of change in behavior over time. For a complete summary of effects on Horizontal Position refer to Table 2.

3.2. Effect of Se supplementation on gene expression

Although we analyzed expression data for each gene with the same statistical model, the patterns of significance varied dramatically among genes. We therefore report the results of our ANCOVA for each gene in Table 3, and highlight the significant results for each gene below.

We observed a highly significant effect of Strain ($F_{3, 98} = 35.62$, $P < 0.0001$) on expression levels of *sepp1a* in the brain of the fish on the control diet. The G and N strains had lower expression levels of *sepp1a* than the S and T strains (the same pattern we observed in our unpublished preliminary microarray study). We also observed a strain-specific response of *sepp1a* expression to Se supplementation, as indicated by a significant interaction of Diet and Strain ($F_{6, 98} = 3.34$, $P = 0.0049$). Specifically, the N strain increased expression of *sepp1a* with both forms of Se supplementation (Fig. 3). Expression of *sepp1a* did not significantly differ among control and supplemented diets in the S, T, or G strains. There was also an effect of Diet ($F_{2, 98} = 3.64$, $P = 0.0298$) on *sepp1a* expression; however the significant difference was not between the control and the supplemented diets, but between the selenomethionine and sodium selenite diets.

We observed a highly significant effect of Strain ($F_{3, 96} = 14.63$, $P < 0.0001$) on expression of *gpx3* in fish fed the control diets. The T strain was significantly lower in *gpx3* expression than all other strains (Fig. 4), a pattern opposite to that observed with *sepp1a*.

Expression of *secp43* significantly increased in response to Se supplementation ($F_{2, 96} = 8.53$, $P = 0.0004$). This effect of Diet was strain-specific, which we observed as a significant Diet by Strain interaction ($F_{6, 96} = 2.49$, $P = 0.0276$) on expression of *secp43*; the N strain significantly increased in expression when fed both forms of Se and the T strain significantly increased in expression when fed the selenomethionine diet (Fig. 5). There was also a marginal effect of Strain ($F_{3, 96} = 2.62$, $P = 0.0549$) on *secp43* expression.

We observed a significant difference in expression of *txnr1* between strains ($F_{2, 62} = 9.83$, $P = 0.0002$), with the G strain showing a significantly higher expression of *txnr1* than the N and S strains. A significant sex-specific diet effect was also observed ($F_{2, 62} = 3.47$, $P = 0.0372$); sodium selenite supplementation in males increased *txnr1* expression relative to the control. Each strain within sex responded differently to Se supplementation as indicated by a complex interaction of Strain, Diet, and Sex ($F_{4, 62} = 3.56$, $P = 0.0112$). In females, supplementation with sodium selenite decreased expression of *txnr1* relative to the control in the G strain (Fig. 6a). In males, *txnr1* expression increased in the G strain with both forms of Se, and increased with sodium selenite supplementation in the N strain relative to the control diet (Fig. 6b). As described in the Materials and methods, the T strain was excluded from the analysis of *txnr1* expression due to the male-biased sex ratio in this strain.

Expression of *dio2* was significantly higher in males than females (Fig. 7a; $F_{1, 61} = 79.32$, $P < 0.0001$). We also observed a significant strain-specific effect of diet on expression of *dio2* (Fig. 7b; $F_{4, 61} = 2.61$, $P = 0.0438$); when supplemented with either Se diet expression of *dio2* increased in the N strain. The T strain was excluded from the analysis of *dio2* expression due to the male-biased sex ratio.

4. Discussion

4.1. Se supplementation elicits a behavioral response in zebrafish

Zebrafish strains fed the control diet varied in their horizontal and vertical swimming depths within the tank. This behavioral variation may be due to adaptation to captivity (Reinhardt, 2001; Álvarez and Nicieza, 2003; Robison and Rowland, 2005; Oswald and Robison, 2008), or it may represent pre-existing variation among the wild populations from which the strains were derived (Álvarez and Nicieza, 2003; Wright et al., 2003; Robison and Rowland, 2005). In response to increased Se, all zebrafish strains increased in Horizontal Position (the proportion of time nearest the observer) with supplementation from both forms of Se, while strain influenced the direction and magnitude of change in Vertical Depth as well as the form of Se that affected the behavior. The observed behavioral response to Se supplementation may be a result of sub-lethal Se toxicity, recovery from a low or deficient Se status, or modification of a conditioned feeding response.

When developing the Se supplemented diets, our goal was to achieve a Se level of 6.0 ppm, which is above the level in the zebrafish's standard diet (*Artemia nauplii*: 0.61 ppm, flake food: 1.1 ppm) and below a toxic Se level. A toxicity threshold for dietary Se has not been determined in zebrafish and the threshold in other fish species is controversial (DeForest et al., 1999; Hamilton and Dill, 2002; Hardy et al., 2010). Hamilton (2003) argues that the Se literature supports a general dietary Se toxicity threshold of 3 ppm for fish, while DeForest et al. (1999) suggest that this is too strict and sets the threshold for warm water fish (such as zebrafish) to 10 ppm dietary Se. Hamilton (2004) does note that there is clear evidence for

species-specific thresholds for dietary Se toxicity. In this study, we did not observe any of the signs of Se toxicity, including abnormal swimming behavior, cessation of feeding, lethargy, and mortality, that have been observed in studies assessing the toxicological implications of Se in fish (Hodson and Hilton, 1983, 2002; Cleveland et al., 1993; Teh et al., 2004; Tashjian et al., 2006). For this reason, we argue that the Se levels in the selenomethionine (7.1 ppm Se) and sodium selenite (5.6 ppm Se) diets are nutritionally relevant for Se supplementation in adult zebrafish, and that the behavioral changes resulting from Se supplementation were not due to toxicity.

If the Se status of our zebrafish was below trace or moderate concentrations before supplementation, it is possible that the behavioral changes we observed were the result of a physiological recovery from Se deficiency (Cohen et al., 1989; Takahashi, 2002; Venardos et al., 2004). At trace and moderate concentrations, Se is required for normal growth, development, and maintenance of homeostatic functions (Hamilton and Dill, 2002). The response in Horizontal Position to Se supplementation may suggest a decrease in the stress or anxiety-like behaviors associated with Se deficiency, such as that seen in rodents and humans (Boylan et al., 1990; Watanabe and Satoh, 1995; Finley and Penland, 1998; Rayman, 2000; Benton, 2002; Shor-Posner et al., 2003). If the observer is viewed as a potential threat, the increase in orientation towards the observer with Se supplementation could be interpreted as a reduction in predator avoidance (Álvarez and Nicieza, 2003). The variation among strains in Vertical Depth also suggests that population influences the behavioral response to Se, which may be the case in humans as well (Hawkes and Hornbostel, 1996; Finley and Penland, 1998; Rayman, 2000; Benton, 2002; Shor-Posner et al., 2003; Rayman et al., 2006). We suggest that the change in behavior in response to Se supplementation indicates that Se homeostasis in the brain is important in regulating behavior in zebrafish and that variation in brain Se homeostasis among populations may influence the response to Se supplementation. However, it is important to note that data on the Se status of these animals are not available, and additional work is required to test whether strains that responded to Se supplementation were indeed Se deficient.

If the zebrafish used in this study were initially deficient in Se, increased appetite may also be a factor in the behavioral changes observed during Se supplementation. A study in Se-deficient chicks, showed an increase in short-term voluntary feed consumption when supplemented with Se (Bunk and Combs, 1980), however, this was not repeated in a long term Se supplementation study using Se-deficient Angora goats (Wichtel et al., 1996). If Se supplementation caused an increase in appetite of the zebrafish, this would likely have resulted in an increase in the time the fish spent at the front of the tank and at surface of the water in anticipation of food.

Also, since behavior was collected immediately prior to the daily feedings the changes in behavior may be interpreted as a conditioned feeding response to the presence of the observer. Studies have shown evidence of associative learning and memory in zebrafish (Bilotta et al., 2005; Sison and Gerlai, 2010). However, even if the changes in behavior were the result of a conditioned feeding response, the key finding in this study is that the behavioral response of the fish fed the Se diets was different than that of the fish fed the control diet and that the change in behavior varied by strain.

4.2. Se supplementation elicits transcriptional changes of selenoprotein genes in the brain

Our data also support our prediction that Se supplementation elicits transcriptional changes of selenoprotein genes in the zebrafish brain. Se supplementation increased expression of the selenoproteins *sepp1a*, *secp43*, *dio2*, and *txnrd1* in the zebrafish brain. This is a key finding because, although selenoprotein expression has been characterized in the brain (Zhang et al., 2008), transcriptional changes in the brain in response to Se are rare.

Expression of *gpx3* did not change in response to increased Se. A hierarchy among selenoproteins with respect to Se supply may explain why *gpx3* did not respond to Se supplementation in the zebrafish brain. Variation among selenoproteins and tissues in response to Se supply has been observed (Schweizer et al., 2004; Hesketh, 2008), and it is possible that the selenoproteins *sepp1a*, *secp43*, *dio2*, and *txnr1* have priority in the brain, causing them to respond first to Se supplementation.

The hierarchy of response among selenoproteins to Se exposure may also be explained by variation in the 3' untranslated region (3' UTR) of selenoprotein genes (Hesketh, 2008). Selenoproteins are so named because they incorporate Se as the amino-acid selenocysteine (Sec) (Schweizer et al., 2004; Hesketh, 2008). Sec is incorporated co-translationally via the recoding of a UGA codon, which usually codes for the termination of translation. The recoding of the UGA codon requires a specific stem-loop structure (Sec-insertion sequence, SECIS), which is located in the 3'UTR in eukaryotic mRNAs. Sec is then incorporated through a complex interaction of the SECIS element, a selenocysteyl-tRNA, two selenophosphate synthetases, and a number of other specific RNA binding proteins. Selenoproteins vary in the length of the 3'UTR and in the position of the Sec-encoding UGA (Hesketh, 2008). Thus, Sec incorporation among different selenoproteins occurs across a range of UGA-SECIS distances, and may influence the response of selenoproteins to Se. Alternatively, the effects of Se on *gpx3* may also occur post-translationally (Toyoda et al., 1990), rather than at the level of mRNA.

4.3. Populations that vary in basal levels of selenoprotein expression in the brain will vary in behavioral and transcriptional responses to Se supplementation

Selenium supplementation significantly influenced both behavior and expression of selenoprotein genes in the brain, but the magnitude and direction of the effect, as well as the form of Se that caused it, varied among strains. The strain-specific effects of Se supplementation on Vertical Depth and on expression of *sepp1a*, *secp43*, *dio2*, and *txnr1* support our hypothesis that variation among study populations can influence the behavioral and transcriptional responses to Se supplementation. To our knowledge, there have been no other studies that compared the transcriptional or behavioral responses to Se administration between multiple populations in either mammalian or non-mammalian research subjects. However, similar population by diet interactions has been observed with responses to toxic metals in rodents, such as behavioral responses in mice to prenatal exposures to methylmercury (Egan et al., 2009).

Interestingly, we observed that expression of *sepp1a*, *secp43*, and *dio2* only increased in the strains with the lowest control expression levels; *sepp1a* and *dio2* expression increased in the N strain (Figs. 3 and 7b) and *secp43* expression increased in the N and T strains (Fig. 5). This suggests that the basal (control) selenoprotein status of the animal may influence the transcriptional response to increased dietary Se. A comparable observation was made in studies measuring mood changes during Se depletion and supplementation in humans. A greater change in mood was observed in subjects with a lower initial Se status (Hawkes and Hornbostel, 1996; Benton, 2002).

Genetic variants in the coding or regulatory regions of selenoproteins or the proteins involved in their synthesis may underlie variation among populations in response to Se administration. SNPs have been identified in the coding and regulatory regions of selenoproteins in humans (Méplan et al., 2007, 2008) and malignant mesothelioma cells (Apostolou et al., 2004), and variation in response to changes in Se status were observed among genotypes. Further, several functional SNPs have been identified in selenoproteins and the selenoenzymes involved in their synthesis that are associated with susceptibility to disease or with baseline mRNA and protein levels (Méplan et al., 2006; Hesketh, 2008). The

variation in behavioral and transcriptional responses between the zebrafish strains in this study may therefore be explained by as yet unidentified SNPs in selenoprotein genes or the proteins involved in their synthesis.

An unexpected finding of our study was the differences in transcriptional response to Se supplementation between males and females. While the exact mechanisms that may regulate this difference are unclear in zebrafish, other studies have reported sexually dimorphic transcriptional responses to nutritional manipulation in this model (Robison et al., 2008). Interestingly, Méplan et al. (2007, 2008) also observed complex interactions between genotype, sex, and BMI in the physiological response to Se supplementation.

5. Summary

Our observations of population-specific responses to Se supplementation in zebrafish, combined with the existence of genetic variation in human Se metabolism (Méplan et al., 2006, 2007), support the hypothesis that variation among study populations can influence the behavioral and transcriptional responses to Se supplementation. This suggests that the conflicting results among studies on the behavioral effects of Se supplementation in humans (Hawkes and Hornbostel, 1996; Finley and Penland, 1998; Benton, 2002; Shor-Posner et al., 2003; Rayman et al., 2006) may result in part from genetic variation within and among the study populations. However, the mechanisms underlying variation in behavioral and transcriptional responses to Se supplementation among populations remain unknown and will require further study to elucidate.

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Fig. 1.

Strain specific response of Vertical Depth behavior to Se supplementation in zebrafish. Plotted values are least square means $\pm$ standard errors. Asterisks (*) indicate significant differences from control values within each strain. Zebrafish strain abbreviations: Gaighatta (G), Nadia (N), Scientific Hatchery (S), TM1 (T). Diet abbreviations: selenomethionine (SeMet), and sodium selenite (NaSel).

Fig. 2. Increase in Horizontal Position behavior (proportion of time nearest the observer) with Se supplementation in zebrafish. a) There was a significant effect of strain on Horizontal Position. b) Both forms of Se supplementation significantly increased Horizontal Position. Different letters denote significantly different least square means ($\pm$ standard errors). Refer to Fig. 1 for abbreviation definitions.

Fig. 3. Strain specific effects of Se supplementation on brain expression of selenoprotein P1a (*sepp1a*) in zebrafish. Plotted values are least square means $\pm$ standard errors. Asterisks (*) indicate significant differences from control values within each strain. Refer to Fig. 1 for abbreviation definitions.

Fig. 4. Strain variation in brain expression of glutathione peroxidase 3 (*gpx3*) in zebrafish. Different letters denote significantly different least square means ($\pm$ standard errors). Refer to Fig. 1 for abbreviation definitions.

Fig. 5. Strain specific effects of Se supplementation on brain expression of tRNA selenocysteine associated protein 1 (*secp43*) in zebrafish. Plotted values are least square means $\pm$ standard errors. Asterisks (*) indicate significant differences from control values within each strain. Refer to Fig. 1 for abbreviation definitions.

Fig. 6. Effect of sex and strain on brain thioredoxin reductase 1 (*txnrd1*) expression in zebrafish. a) For females, there was a strain specific effect of Se supplementation. Note that the T strain was removed from the analysis due to a male-biased sex ratio in the strain. b) For males, there was also a strain specific effect of Se supplementation. Asterisks (*) indicate significant differences from control values within each sex. Refer to Fig. 1 for abbreviation definitions.

Fig. 7.

Effect of sex and strain on brain iodothyronine deiodinase 2 (*dio2*) expression in zebrafish. a) Male zebrafish had significantly higher brain expression of *dio2* than females. Different letters denote significantly different least square means. Note that the T strain was removed from the analysis due to a male-biased sex ratio in the strain. b) Se supplementation significantly increased brain *dio2* expression in the N strain. Asterisks (*) indicate significant differences from control values within each strain. Refer to Fig. 1 for abbreviation definitions.

Table 1

Primer sequences (F = forward, R = reverse) used for quantitative Real-Time PCR assays of gene expression.

Gene	Primer	Sequence
Selenoprotein P 1a	<i>sepp1a</i> zf F	ATCCTGAGCCACCCACAT
	<i>sepp1a</i> zf R	ACTCTTCAAGTTGCGCTGAA
Glutathione peroxidase 3	<i>gpx3</i> zf F	TCCAGGAAATGGATTTCGTTC
	<i>gpx3</i> zf R	TCTCTCCTACAGGCGGACAT
Thioredoxin reductase 1	<i>txnrd1</i> zf F	CGGAGCTGCTATGAAGTGTG
	<i>txnrd1</i> zf R	AGCCGGATTGAGTGATGTCT
Iodothyronine deiodinase 2	<i>dio2</i> zf F	ACTCGGACACTTGGCTTGAC
	<i>dio2</i> zf R	CAAATGAGCGACTGCCTATG
tRNA selenocysteine associated protein 1	<i>secp43</i> F	GGGCGATTTAGACCCATACA
	<i>secp43</i> R	CCAGCTGAGCCTCCAGTAAC
Eukaryotic elongation factor 1 α	<i>ef1a</i> F	GTACTACTCTTCTTGATGCCC
	<i>ef1a</i> R	GTACAGTTCCAATACCTCCA

Table 2

Results of ANOVA for behavioral data (P-value shown for each variable measured).

Model term	Behavior	
	Vertical Depth	Horizontal Position
Diet	0.1450	0.0070
Strain	<0.0001	<0.0001
Strain*Diet	<0.0001	0.5261
Sex	0.0181	0.6743
Day	<0.0001	<0.0001
Strain*Day	0.1470	0.0362
Diet*Day	0.5195	0.3132
Strain*Diet*Day	0.2923	0.7993

Table 3

Results of ANCOVA for gene expression data (P-value shown for each variable measured).

Model term	Gene					
	<i>sepp1a</i>	<i>gpx3</i>	<i>secp43</i>	<i>txnr1</i>	<i>dio2</i>	
Diet	0.0298	0.1951	0.0004	0.6747	0.2385	
Strain	<0.0001	<0.0001	0.0549	0.0002	0.2319	
Strain*Diet	0.0049	0.5453	0.0276	0.3934	0.0438	
Sex	0.5107	0.8936	0.7138	0.1441	<0.0001	
Diet*Sex	0.5270	0.9335	0.6907	0.0372	0.7423	
Strain*Sex	0.6448	0.9658	0.9677	0.2536	0.4846	
Strain*Diet*Sex	0.4328	0.9699	0.9788	0.0112	0.4716	