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Effects of Global DNA Methylation Changes on Neurobehavior in Zebrafish

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EFFECTS OF GLOBAL DNA METHYLATION CHANGES ON NEUROBEHAVIOR IN ZEBRAFISH

by

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ABSTRACT

EFFECTS OF GLOBAL DNA METHYLATION CHANGES ON NEUROBEHAVIOR IN ZEBRAFISH

by

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Under the Supervision of Professor Michael J Carvan, III

A number of environmental neurotoxicants modulate DNA methylation, but its influence on neurobehavior remains unclear. The laboratory has established that low-level developmental methylmercury exposure induces neurobehavioral deficits; the current results demonstrate that it also induces global DNA hypomethylation. *DNA methyltransferase 1*-mutant zebrafish (exhibit ~70% reduction in enzymatic activity) were used to assess the role of DNA hypomethylation on behavior. Several neurobehavioral assays including the C-start escape, circadian rhythm, basic locomotion and visual-motor response (VMR) were also performed. There was a significant difference in VMR between the wild type and mutant animals. Other behavior assays revealed no significant difference, primarily due to small sample size, but several trends were observed. Mutants demonstrated a lack of persistent circadian rhythms when held in constant low light, and were hyperactive under normal lighting conditions. In conclusion, toxicant-induced global hypomethylation of DNA may alter neurobehavior in morphologically normal eleutheroembryos and the mechanism needs further investigation.

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INTRODUCTION

Embryonic development is a highly complex process which requires epigenetic regulation (*e.g.*, DNA methylation, histone modifications, non-coding RNA, and nucleosome positioning [Jackson *et al*., 2002; Barr and Misener, 2015; Englander *et al*., 1993]) of gene expression (Cedar, 1988). Epigenetics is defined as regulation of gene expression by external and/or environmental factors instead of inherent genetic mutations (Robertson and Wolffe, 2000). For example, DNA methylation is an epigenetic modification that plays a major role in gene silencing and genomic stability (Jirtle and Skinner, 2007). Dysregulated DNA methylation has been reported as a potential etiological mechanism in human cancers (Vo and Millis, 2012; Avraham *et al*., 2014; Zhoa *et al*., 2015) and has been strongly linked with many other types of human disease (Ritter *et al*., 2015; Reynolds *et al*., 2015). Thus, elucidating and understanding the mechanisms by which DNA methylation influences physiology, development, and disease has become an important research focus.

Approximately 60-90% of all CpG nucleotidesin mammalian DNA are methylated (Tucker, 2001), while in zebrafish ~20% are methylated (Fang *et al*. 2010). However, CpG islands (*i.e.*, regions of DNA which tend to include the transcriptional promoter site for genes) are typically unmethylated (Illingworth and Bird, 2009). The level of transcription is dependent on the level of DNA methylation at the promoter CpG island (Fig. 1).

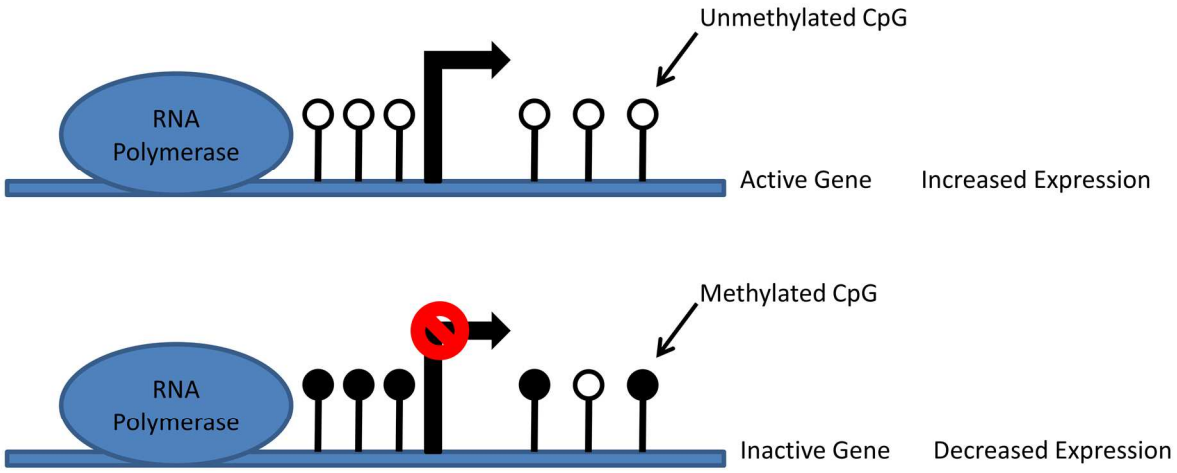

Fig. 1. DNA methylation and gene transcription. Increasing methylated cytosines, especially at promoter sites, decreases gene transcription. DNA methylation plays a key role in gene silencing and epigenetic regulation.

A global increase in DNA methylation inhibits gene transcription and a decrease in DNA methylation activates gene transcription. DNA methylation affects the ability of transcriptional regulatory proteins (*e.g.*, transcription repressors, MeCP1 and MeCP2 [Martin *et al*., 1999]) to bind the DNA. Mechanistically, DNA methylation is the addition of a methyl group to the fifth carbon atom a sixth carbon cytosine in a CpG dinucleotide (Goll and Helpern, 2011) via DNA methyltransferases (DNMTs; Martin *et al*., 1999), which have been found to be homologous among many vertebrates (Bestor, 2000). The methyl group required for this biochemical reaction originates from S-adenosylmethionine (SAM), the primary methyl donor in the methionine synthesis pathway (Loenen, 2006). The most abundant DNMT in vertebrates is *DNA methyltransferase 1* (DNMT1), which contributes greatly to the establishment of methylation patterns in mitotic cells (Li *et al*., 2007). During DNA replication, DMNT1 is recruited to the replication fork by *ubiquitin-like, containing PHD and RING finger domains, 1* (UHRF1), whereby

DNMT1 methylates the nascent hemi-methylated DNA (Bostick *et al*., 2007). Studies have shown that the complete loss of DNMT1 function yields embryonic lethality in mice (Oakes *et al*., 2007) and mortality in zebrafish after 8 days post fertilization (DPF) (Anderson *et al*., 2009). In the methionine pathway (Fig. 2), the enzyme *methionine synthase* synthesizes methonine from homocysteine by the use of methyl groups donated from methylcobalamin, folic acid

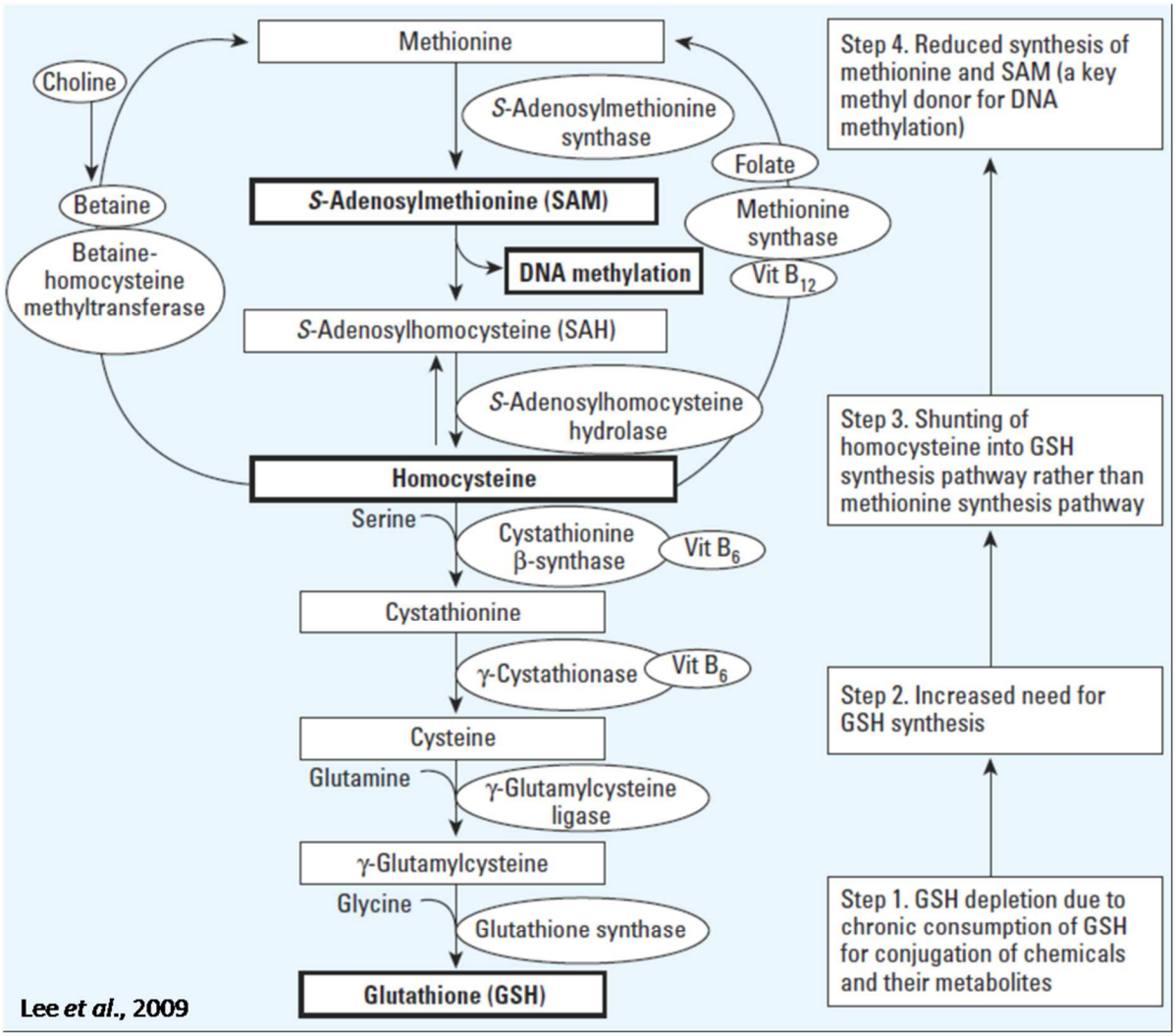

Fig. 2. A diagram illustrating the hypothesis that methionine pathway can explain how environmental toxins can mechanistically cause DNA hypomethylation. During chronic toxin exposure, glutathione is depleted and the need for more shifts the methionine pathway in order to synthesize more glutathione. This shunts the production of methionine that will later be catalyzed to s-adenosylmethinone, limiting DNA methylation. Image taken from *Environmental Health Perspectives: http://ehp.niehs.nih.gov/0900741.*

(folate), choline and betaine (Cooney, 1983). Once the methyl group is removed from SAM, s-adenosylhomocysteine can be recycled back to homocysteine and the methionine cycle beings again. Homocysteine normally cycles through the methionine pathway; however, it also contributes to glutathione synthesis, which is needed to maintain redox balance. In the case of toxin exposure, glutathione, the primary antioxidant and free radical scavenger in the cell, can become depleted. Lee *et al*. (2009), hypothesized that during toxin exposures, the pathway shifts to generate more glutathione to compensate for its depletion and maintain redox balance. If this occurs during early embryogenesis, it is possible that methionine synthesis from homocysteine consequently decreases, which, in turn, limits the ability of a cell to appropriately regulate DNA methylation during rapid mitotic divisions. In addition, Deth *et al*. (2008) demonstrated that cellular oxidative stress can suppress the methylation cycle by inhibiting the folate-and cobalamin-dependent pathways of methionine synthase. Collectively, this evidence supports that further research focused on the effect of environmental insults on DNA methylation and subsequent phenotypic expression is a necessity.

Many studies have shown correlations between exposure to environmental toxicants (*e.g.*, mercury, lead, bisphenol A, *etc*.) and DNA methylation changes that may lead to the transgenerational inheritance of diseases (Bose *et al.*, 2012; Hanna *et al*., 2012). Methylmercury (MeHg) is a ubiquitous environmental pollutant that bioaccumulates rapidly in fish and shellfish (Nyland *et al*., 2011). It is neurotoxic and impacts the abilities of animals to sense and respond to environmental changes (Pilsner *et al*., 2010). Acute MeHg exposure results in varying degrees of neuronal loss in the cortex, cerebellar granular cells, primary motor cortex and peripheral nerves (Heath *et al*., 2010). A study by Pilsner *et al*. (2010), found

a decrease in DNA methylation with increasing methylmercury concentrations in the brain stems of polar bears. Polar bears are apex predators that are known to feed on high mercury diets in the wild. In certain regions of in the world, humans are also known to consume a lot of high mercury diets (Nyland *et al*., 2011). If DNA methylation alterations occur from environmental toxicants and such impacts have been shown in an organism such as polar bears, then multi-organism effects should be investigated.

Altered gene expression due to altered DNA methylation has been shown in numerous studies of cancers and mental disorders (*e.g.*, schizophrenia; Abdolmaleky *et al.,* 2014). In the case of cancers, typically DNA methylation is increased at promoter sites, while globally the DNA is hypomethylated (Galm *et al*. 2006). If a gene, such as a tumor suppressor is silenced from hypermethylation of the promoter region, then the potential for cancers to thrive increase (Esteller, 2003). Other important genes that show correlation to cancers from hypermethylation are O6-methylguanine–DNA methyltransferase (MGMT; Esteller *et al*., 2000) involved in DNA repair and cyclin-dependent kinase inhibitor 2B (CDKN2B) involved in cell cycle regulation (Herman *et al*., 1997). While many diseases occur directly as the result of direct toxicant exposure, it is also suggested that inherited diseases can also be found in future generations without subsequent exposure and that the mechanism is directly related to DNA methylation (Guerrero-Bosagna and Skinner, 2012). Heavy metals such as MeHg (Bose *et al*. 2012), lead (Pilsner *et al*., 2009), and cadmium (Jiang *et al*., 2008) have been shown to cause methylation changes in multiple organs. Bose *et al.* (2012) were able to demonstrate *in vitro* that MeHg-induced DNA hypomethyaltion in neural stem cells (NSC) was maintained in

daughter cells three cell divisions after MeHg was removed. Investigating the epigenetic mechanisms by which environmental toxicants cause disease is essential.

In this study, we investigated the effects MeHg on of global DNA hypomethylation, and the effects of DNA hypomethylation on the neurobehavior of developing zebrafish. We considered a number of options including drug-induced DNA hypomethylation to emulate the potential impact of known environmental toxicants. 5-Azacytidine (5-aza) is a chemotherapeutic agent that binds to the catalytic domain (CXXC) of DNA methyltransferase enzymes and prevents DNA methylation (Santi *et al*., 1983). Pharmacologically induced DNA hypomethylation in zebrafish eleutheroembryos is known to cause loss of tail, abnormal somite patterning, cranial abnormalities, and whole or partial loss of differentiated notochord (Martin *et al*., 1999). These deformities will occur more frequently if an embryo is exposed during specific critical developmental windows. Just after fertilization, there is a dramatic decrease in global DNA methylation followed by re-methylation and establishment of the normal methylation pattern that is dependent on each organism's zygotic genome activation (Mhanni and McGowan, 2004). For zebrafish embryos, DNA methylation patterns can be altered from fertilization until the blastocyst stage. If 5-aza is used to induce DNA hypomethylation during this critical window of development, then it is possible it will cause lifelong DNA methylation abnormalities in zebrafish.

Contrary to DNA hypomethylation, supplementations with methyl donors have been shown to increase DNA methylation. Waterland and Jirtle (2004) have shown that dams supplemented with additional folic acid were able to change the methylation pattern of mutant A^{vy} alleles causing a significant decrease in the amount of obese mice. Medici *et al*. (2014) were

able to induce DNA hypomethylation and specific gene transcript changes that were corrected with betaine supplementation *in utero* in cystathionine beta synthase (CβS) heterozygous C57BL/6J mice. Although there are many publications showing that methyl donors are imperative to normal development (Sun *et al*., 2009; Finkelstein *et al*., 2015; Xu and Sinclair, 2015), little is known about the mechanism(s) whereby they induce methylation changes during xenobiotic exposure.

Studies using zebrafish as a model organism have steadily increased in recent decades, with this model organism being exploited in many fields of biomedical research, including toxicology, to study processes related to disease susceptibility, behavior and risk (MacPhail *et al*. 2009).

Behavior is a useful tool for assessing neurological development in zebrafish as a wide range of neurobehavioral assays were developed to screen for mutant phenotypes and are generally well-developed. Behaviors that have been investigated include sensory and motor functions (Burgess and Grana to 2007), synaptic transmission and plasticity via the c-start response (Kimmel *et al*. 1974), chronobiological effects via circadian rhythm (Rosenwasser *et al*. 2014) and image and motion processing by the optomotor response (Orger and Baier, 2005). Dysfunction in a specific behavioral assay can lead to mechanistic investigations of environmental toxicants.

The C-start is an escape behavior seen in fish, larval frogs and toads, which has a very distinct and well understood neural circuit (McLean *et al*., 2000; Kimmel *et al*., 1974). The C-start is important for predation escape in many vertebrae species (Eaton *et al*., 1977).The primary cell responsible for this escape response is the Mauthner cell, the largest neuron in the

central nervous system of fish (Kimmel *et al*., 1981). In the zebrafish model, the Mauthner cells develop during gastrulation, around 8 HPF and form in the rhombomere 4 region of the hindbrain (Mendelson, 1986). It is possible to evoke the C-start response by different stimuli that act through the trigeminal nerve and the acoustic-vestibular system, which functionally develop around 48 HPF (Kimmel *et al*., 1990; Foreman and Eaton, 1993). Additionally, touching the tail elicits a C-start escape response through activation of one or more Rohon-Beard sensory neurons which then activate the glutamatergic dorsolateral commissural sensory interneuron (Easley-Neal *et al*., 2013; Li *et al*., 2003). When these sensory neurons are activated via a tactile stimulus, they also activate one Mauthner cell by glutamatergic chemical signaling (Ali *et al*., 2000). This signal travels down the spinal cord to primary motor neurons on side opposite the stimulus (Sillar and Roberts, 1988). There are six to eight primary motor neurons in the dorsal motor column per spinal cord segment (Eisen *et al*., 1986; Moreno and Ribera, 2009) and many smaller, secondary motor neurons located more ventrally in the motor column (Menelaou and McLean, 2012). In zebrafish, these motor neurons stimulate slow-tonic-redand fast- twitch- white muscles fibers (Fetcho, 1992). The entire C-start response, from the Mauthner cell excitation through muscle contraction, occurs within 2ms (Zottoli, 1977). There is also an inhibitory feedback system to ensure that only one Mauthner cell fires in order for the animal to escape in the opposite direction of the stimulus (Eaton and Kimmel, 1980). In addition to the C-start response, circadian rhythm is another advantageous endpoint that permits the evaluation of the potential chronobiological effects of DNA methylation on neurobehavior.

Circadian rhythm is an important biological clock that is controlled by the suprachiasmatic nucleus (SCN) in the mammalian brain (Rosenwasser *et al*., 2014), that controls daily functions and energy levels via metabolism and cell cycle (Li *et al*., 2015). In this system, one factor that commonly entrains the behavioral circadian rhythm is light. Light enters the eyes and travels to the photoreceptors in the retina to form an image. In the retina, there are specialized intrinsically photosensitive retinal ganglion cells (ipRGCs) that use melanopsin to send a signal directly to the SCN by means of the retinohypothalmus tract (Hatter *et al*., 2002; Gooley *et al*., 2001). These ipRGCs are non-image forming and do not require functional cones or rods to entrain the circadian rhythm (Freedman *et al*., 1999). The signal is then sent through long axons to the brain and cross at the optic chiasm. Here, the signal can then either continue to the back of the brain, or divert upward towards the SCN (Berson *et al*., 2002). Once entrainment is complete in the SCN, the rhythm is communicated to the pineal gland where melatonin is produced (Steele *et al*., 2006). In mammals, the SCN is the key oscillator in regulating the circadian rhythm; however, in teleosts, other oscillators can regulate the circadian clock in the absence of SCN. For example, the mutant *cyclops* (*cyc*) zebrafish lack a ventral brain and SCN, but still produce a circadian rhythm (Noche *et al*. 2011). A set of experiments by Whitmore *et al*., (1998, 2000) showed that the heart and kidneys of zebrafish have independent circadian rhythm oscillators, which remained active in culture. In mammals, the circadian rhythm has been extensively investigated; however, in zebrafish the molecular mechanism controlling circadian rhythm is still under investigation.

The effect of visual stimuli on locomotor functions is another area of behavioral assessment that is gaining ground as a powerful tool in neurobehavior. In general, locomotion

plays an integral role in feeding, social, and defensive activities throughout the zebrafish lifespan (Colwill and Creton, 2011). The optomotor response (OMR) is an innate behavior that develops 5 DPF in the zebrafish. When larva is presented with a whole-field moving visual stimulus, it will turn and swim in the direction of perceived motion (Portugues and Engert, 2009). The OMR is initiated by red and green cones (Orger and Baier, 2005); however exactly which retinal ganglion cells are involved remain unclear (Roeser and Baier, 2003). Twelve neurons have been found in both sides of the hind brain that transmit signals down the spine for a motor response to occur (Orger *et al*., 2008). Another frequently used behavioral assay is the visual-motor response (VMR), where locomotion changes are induced by changes in light (Emran *et al*., 2008). In the case of the VMR, zebrafish have been found have increased locomotor activity during sudden removal of light and decreased locomotor activity when lights are suddenly turned on (Prober *et al*., 2006). The circadian rhythm plays an integral role in daily locomotor activity, but can be overridden by sudden changes in light stimuli (Burgress and Granato , 2007). Interestingly, Burgress and Granato, (2007) have also reported that the Mauthner cell is not required for the VMR. In summary, if neurocircuits are indeed disrupted, the exploitation of a suite of neurobehavioral assays is a comprehensive approach that may elucidate the potential mechanism(s) of an observed phenotype.

In this study, we investigated the effects of MeHg on global DNA hypomethylation, and the effects of global hypomethylation on zebrafish neurobehavior. We first compared levels of DNA hypomethylation from methylmercury and 5-aza exposures. Then, we investigated several neurobehavioral phenotypes induced by toxin exposures. We completed the experiment by using *dnmt1* S^{872} strain zebrafish as a model for global hypomethylation as both heterozygous

and homozygous mutant individuals have reduced DNA methylation compared to wild-type individuals (Anderson *et al*. 2009). By using the mutant zebrafish, we are able to establish direct interactions between neurobehavioral phenotypes and DNA hypomethylation in the absence of toxin exposure. We hypothesize that global reduction in DNA methylation impacts zebrafish behavior and may play a role in toxicant-induced neurobehavioral abnormalities.

Materials and Methods

Experimental Animals

All studies were conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) at the University of Wisconsin-Milwaukee. For this study, EkkWill (EK) zebrafish (Waterlife Resources, Ruskin, FL, USA) and *dnmt*1 S^{872} (+/-) zebrafish (ZFIN ID: ZDB-GENO-100112-13) were used (Anderson *et al*. 2009). Zebrafish were maintained in plastic aquaria (1.5-39L) at a density of ≤ 10 fish/L in 28°C dechlorinated tap water with a 14:10hour light:dark photoperiod. Embryos were raised in E2 medium (248.5mM NaCl, 9.56mM KCl, 18.9mM $MgSO_4$, 2.15mM KH_2PO_4, 0.08mM Na2HPO4, 24.8mM $CaCl_2$, and 13.6mM $NaHCO_3$. Adults were fed a combination of Artemia nauplii (Brine Shrimp Direct, Osden, UT, USA) and Aquarian™ flake food (Aquarium Pharmaceuticals, Inc., Chalfont, PA, USA).

Eleutheroembryo Rearing

Embryos were obtained shortly after spawning and transferred to 100x25 mm Petri dishes (VWR, Radnor, PA, USA). In each dish, 100 embryos were placed in 30mL of fresh E2 medium. At 24 HPF, embryo mortality was assessed and any dishes with >20% mortality were discarded. EK eleutheroembryos were raised and exposed to reagents in the Petri dishes and early life stage toxicity scoring was performed in non-coated Falcon® Multiwell™ 12 well plate (Fisher Scientific, Pittsburgh, PA,USA). For neurobehavioral trials, individual *dnmt*1 S^{872} eleutheroembryos of unknown genotype were placed in 2mL of fresh E2 medium in each well of a non-coated Falcon® Multiwell™ 24 well plate (Fisher Scientific, Pittsburgh, PA, USA, CAS: 08-722-51). Half volume medium changes were performed daily for to maintain eleutheroembryo health. During days of neurobehavioral trials, medium changes were

performed post-trial. Because eleutheroembryos still receive nourishment from the yolk, no supplemental feedings were performed. Physical abnormalities were assessed for each eleutheroembryo and malformed fish were excluded from the neurobehavioral data sets for each assay.

Tracking of Fish Movement

Eleutheroembryos were individually tracked using DanioVision™ (Noldus Information Technology, Leesburg, VA, USA). The experimental settings were selected for round wells of a 24-well plate and parameters of distance(cm) and time (sec) were evaluated. The sample rate was set to a standard of 25 frames per second. The area of each well was adjusted and a template was used to encompass each well and the width of the plate was calibrated to 12.25cm. Detection settings were set for 'differencing' with the subject was selected to be 'darker' than the background with a 'medium/slow' change in background. Subject contour settings were adjusted to increase the capture of the eleutheroembryos. Contour erosion and dilation were both selected and dilation was set at 3 pixels. The option to dilate first, than erode, was also chosen. For the OMR, EK eleutheroembryos were tracked using a digital camera and manual analysis was performed for each trial. All DanioVision™ and OMR trials were performed in a designated behavior and dark room, respectively, with a controlled air temperature of 28°C. EthoVision® XT software (Noldus Information Technology) was used to analyze the trials. Data for the locomotor and VMR were analyzed in velocity (cm/s) in one-minute time bins while the circadian rhythm was analyzed in one-hour time bins. Locomotion, VSR, and circadian rhythm data were presented as mean velocity ± S.E.M (cm/s) for each

designated time interval. Qualitative observation was used to analyze data for the OMR and the data was presented as number of animals that successfully performed the OMR.

5-azacytidine Dose Response Curve

The first preliminary trial was to evaluate the magnitude to which 5-azacytidine (5-aza) can induce DNA hypomethylation in wild-type zebrafish. A dose response curve (*i.e.*, 0, 1, 3, 10, 30, 50 and 100 µM) was performed using 5-aza (Simga-Aldrich, St Louis, MO, USA). Freshly fertilized embryos (1 cell stage) were statically exposed for 24 hours to determine the highest concentration which would cause minimal morphological deformities. Exposures were performed in 12-well plates, 2 mL of E2 medium ± treatment, and 10 eleutheroembryos per well. An early life-stage toxicity score (ELSTS) was used to access the amount of morphological deformities associated with each concentration. A scoring system of 0 to 4 was used to evaluate the ELSTS (0 = no abnormalities, 1 = one abnormality, 2 = two abnormalities, 3 = three abnormalities, and 4 = grossly deformed). Data was analyzed by two-way ANOVA.

DNA Methylation Quantification by MethylFlash™

The MethylFlash™ DNA Methylation Quantification Kit (Colorimetric) (Epigentek , Farmingdale, NY, USA) is an ELISA-like micro-plate assay that uses antibodies to directly measure the amount of 5-methylcytosine (5-mC). Samples were stored at 80°C in 100 µL RNAlater® (Thermo Fisher Scientific, Grand Island, NY, USA). DNA was isolated using the Mini Genomic DNA Mini Kit (Tissue) (IBI Scientific, Peosta, IA, USA) with a modification for single embryo optimization: 200 µL GT buffer was added to each individual eleutheroembryo. First, each sample was treated with 10µL of proteinase K, vortexed, and subsequently incubated at 60°C for 5 to 10 mins. Then, each sample received 200 µL of GBT buffer, vortexed, and

incubated at 60°C for 10 mins. Next, RNase A (2 µl; Qiagen, Valencia, CA, USA) was added; each sample was then vortexed and incubated for 5 mins at room temperature (~20°C). Rinsing was performed per the manufacturer's protocol. Elution buffer was warmed to 60°C during the last centrifugation phase, 30 µL of elution buffer was applied to the column matrix and incubated for 5 mins before the final centrifugation in a new 1.7 mL Eppendorf tube. Sperm DNA isolation was conducted using the same protocol. DNA quantification and quality were assessed using two methods prior to use in the MethylFlash™ assay. Genomic DNA was evaluated in triplicate using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and a Qubit® 2.0 Fluorometer in tandem with a Qubit dsDNA Broad Range Assay Kit (Invitrogen, Carlsbad, CA, USA).

MethylFlash™ was then performed using 100 ng of (RNA free) genomic DNA (8 µL) for each reaction. A duplicate for each sample was made by pipetting 180 µL of ME2 binding solution to 225 ng of genomic DNA in a 200 µL PCR tube. All samples were then immediately vortexed. 80 µL of the mixture + x µL DNA (100 ng) was added to each assay well in randomized pattern. A standard curve (water blank, 5 ng negative control, and 0.25, 0.5, 0.75, 1, or 2 ng positive control DNA) was made in the same fashion as stated above. The positive (100% methylated DNA) and negative (0% methylated DNA) control DNA is provided with the MethylFlash™ kit to help quantify percent 5-mC. All liquids were removed using a vacuum suction system, without touching the bottom of the well. All subsequent steps were performed per the MethylFlash™ protocol. Using a Synergy H4 plate reader (BioTek, Winooski, VT, USA) an absorbance at a wavelength of 450 nm was read immediately after stopping the reaction at a medium blue color as stated in the MethylFlash™ protocol. It is important to note that usage

of the Synergy™ H4 dual reagent injector module is not compatible for use with the MethylFlash™ reagents. A one-way ANOVA was performed using all raw absorbance values for each experimental group to assess potential statistical significance and all control absorbance values were standardized to a value of 1 to evaluate a fold-change comparison. The overall purpose of the MethylFlash™ is to quickly and accurately quantify global DNA methylation.

The first MethylFlash™ experiment was performed using embryos that were exposed to 30-µM 5-aza to access the level of DNA hypomethylation that developed from drug-induced alteration. We also evaluated the effect of one static exposure *versus* a continual exposure. Freshly spawned embryos were collected and exposed to 30 mL of E2 in absence or presence of 30-µM 5-azaat a density of 100 embryos per Petri dish. The 0 µM (control) group and one 30 µM 5-aza group did not receive any medium changes. The second 30 µM 5-aza group received a 30 mL medium change with a fresh mixture of 5-aza every 8 hours. After 24 HPF, DNA was isolated and MethylFlash™ was performed. Statistical analysis was performed by one-way ANOVA.

The second MethylFlash™ experiment was designed to look at the effects of methylmercury on the DNA methylation changes. A methylmercury dose response was performed on freshly fertilized embryos. A dose range (*i.e.*, 0, 0.001, 0.003, 0.01, 0.03, and 0.1 µM) of MeHg was used to statically expose embryos (1 cell stage) to 12 HPF. Absolute ethanol was used as vehicle for the MeHg. Embryos were then collected, DNA isolated, and MethylFlash™ performed. Statistical analysis was performed by one-way ANOVA.

The third MethylFlash™ experiment investigated transgenerational effects of MeHg on EK zebrafish sperm. A F1 generation of EK zebrafish was made by a static exposure does range

as previously mentioned. The F1 generation was allowed to age to adults without further MeHg exposures and the F2 and F3 generations were created. Sperm was collected by gentle abdominal massage and glass capillary tubes (World Precision Instruments, Inc, Sarasota, FL, USA, Item # 1B100F-6) on Tricaine-s (Western Chemical Inc., Ferndale,. WA, USA) anesthetized F1 and F3 adult males from the 0 and 0.01 µM MeHg groups. The sperm was transferred to 100 µL RNAlater in 1.7 mL eppendorf tubes in pools of 3 per sample, and frozen at -80°C. DNA was extracted using the same individual embryo protocol and MethylFlash™ was performed. Statistical analysis was performed by student t-test comparing the 0.01 µM MeHg group to the respective generation control.

Optomotor Reponse

The optomotor response assay was used to investigate the effects of 5-aza on DNA hypomethylation. Cyanocobalamin (B_{12}) (Sigma-Aldrich, St Louis, MO, USA), exposure was also performed to investigate if any hypomethylation-induced phenotype could be rescued. Freshly fertilized EK embryos (1 cell stage) were exposed with 0 µM, 30 µM 5-aza, 1 µM cyanocobalamin, or 30 µM 5-aza + 1 µM cyanocobalamin. Embryos were raised in the absence of light for the first 24 hours, as cyanocoblamain is light sensitive, and then rinsed three times with E2 medium. At 144 HPF, 25 eleutheroembryos from each group were transferred to a Fisherbrand™ square disposable Petri dish with grid (Fisher Scientific, Pittsburg, PA, USA) with 30 mL of E2 medium. A small piece of plastic was used to confine the eleutheroembryos to the far left column of the grid. The dish was placed on a computer monitor showing a still white image (Fig. 3). After a 2 min acclimation period, an animation of 2 cm alternating white and red bars was started in the right direction at a frequency of approximately 2 hz. A video recording

was also performed using a Sony Cyber-shot DSC-HX94 Exmor R CMOS digital camera (Sony Co, New York City, NY, USA). After 5 seconds of the animation, the plastic gate was removed and eleutheroembryos were allowed to free-swim for 1 min. The performance of an animal was deemed acceptable if it traveled in the right direction, moved a minimum of 7 cm out of a maximum possible distance of 9 cm, and performed this action within 45 seconds. Data was analyzed by hand and assessed by one-way ANOVA.

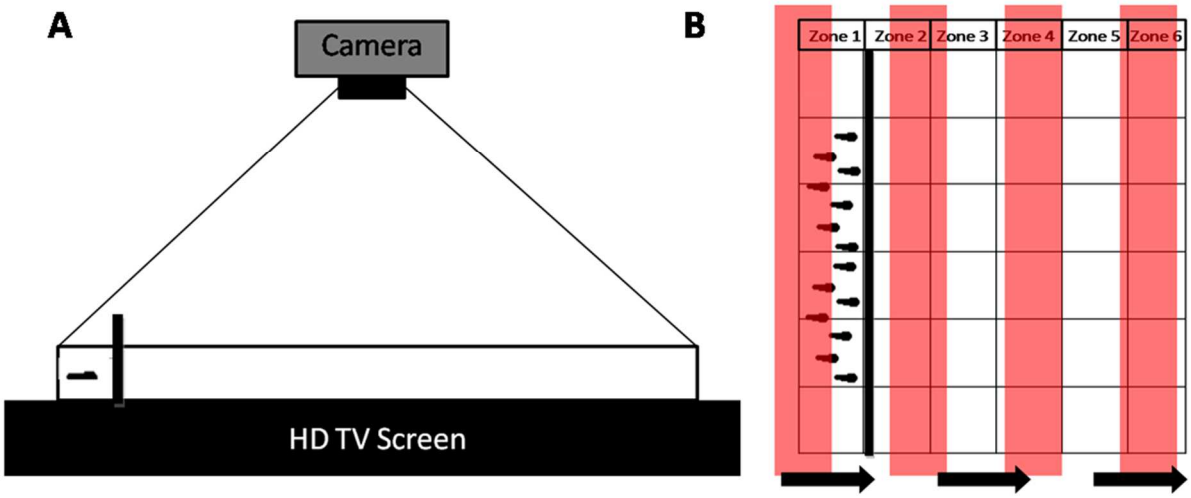

Fig. 3. Schematic depiction of the optomotor response (OMR) used in this study. A. Shows the side view of the OMR apparatus. B. During the OMR, the eleutheroembryos swim in the direction of the moving stimulus. Direction of motion is indicated by arrows.

Touch Response Assay

The touch response assay was utilized to evaluate the synaptic transmission and plasticity of the C-start neurocircuit in individual eleutheroembryos. A "touch" was performed using a 1 mL transfer pipette to gently touch the base of the cranium, near the otolith. A scoring system of 0 to 2 was used to evaluate the quality of escape (0 = no movement, 1 = poor escape, 2 = normal escape). Kruskal-Wallis one-way ANOVA of variance of ranks was used to

determine statistical significance. A second touch response assay was performed at 144 HPF, using identical methods, to further investigate functionality of the more developed neural network and musculature.

Circadian Rhythm

Circadian rhythm trials were used to investigate potential chronobiological disruption within individuals by comparing the time of day to locomotor activity. At 5 DPF, a 24-well plate containing one eleutheroembryo per well, was placed into the DanioVision™. Each trial was initiated at approximately 15:00 hours, using a trial control setting of constant 1% light and a recording time of 56 hours. Medium changes were not performed during the recording to prevent interference of larval behaviors. Data were analyzed in one-hour time bins and a two-way ANOVA was used to determine statistical significance.

Locomotor and Visual-Motor Response

At 144 HPF eleutheroembryos were assessed for basic locomotor activity and visual-motor response. Assays were performed using the DanioVision™ between 12:00 and 16:00 hours each day in 24-well plates, containing one eleutheroembryo per well. Each trial started with a twenty-minute acclimation period in the absence of light. The first trial assessed locomotion of the eleutheroembryos in the absence of light for thirty minutes. The second trial assessed locomotion of the eleutheroembryos after a transition from the dark acclimation phase to 30 minutes of light at intensity of 100% in the DanioVision™. The third trial was an visual-motor response assay and was performed by interchanging cycles of ten minutes of light, ten minutes of dark, for three cycles. Each 24-well plate was only used for one trial type on

each day; and the order of trial type was randomized. Statistical analysis was performed by two-way ANOVA and Kruskal-Wallis one-way ANOVA of variance of ranks.

Genotyping of dnmt1 S^{872}

After completion of the VSR or circadian rhythm neurobehavioral trials, embryos were stored at -80°C. Genotyping was then performed on each individual after all behavioral trials were completed. DNA was isolated using a rapid digest method for PCR (Meeker *et al*. 2007). PCR was performed by the addition of 2 µL of genomic DNA to a 25 µL reaction with the following reagents: 8.5 µL of Hyclone™ Hypure Molecular Biology Grade Water (VWR, Radnor, PA, USA), 1 µL each of 20 µM *dnmt*1 S^{872} specific forward and reverse primers design by Anderson *et al*. 2009, (Forward: 5'-GACACCTACCGCTTCTTTGG-3; Reverse: 5'-TCTCCTGCTCACAGGCTCT-3'), 12.5-µL 2x Promega PCR Master Mix (Promega, Madison, WI, USA, CAS: M7505). Reactions were amplified in a Bio-Rad T-100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) using the following conditions: 95°C for 5-minutes; 45 cycles of 95°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds; followed by 72°C for 3-minutes.

Restriction enzyme digestion was then performed using HincII (New England Biolabs, Ipswich, MA, USA) in a 25 µL reaction using the following reagents: 0.5 µL of HincII, 2 µL 10x 3.1 buffer, 2 µL 10x bovine serum albumin (50mg/ml, Invitrogen, Carlsbad, CA, USA), and 15.5 µL Hypure™ water. Reactions were digested using the following conditions in the Bio-Rad T-100 Thermal Cycler: 37°C incubation for 6 hours; followed by 65°C for 20 minutes to inactivate.

Genotypes were confirmed by running 20 µL of HincII digested product on 2% agarose gel, containing 40mM Tris-acetate and1mM EDTA, at 50 volts for approximately 3 hours on a EC600 power supply (E-C Apparatus Corp, Milford, MA, USA). Then, two µL of loading dye

containing SYBR gold (Invitrogen, Carlsbad, CA, USA) was added to visualize the bands. A 50 bp ladder (New England Biolabs, Ipswich, MA, USA) was used for band size comparison. Confirmation of restriction products was performed by matching products the corresponding genotypes: WT - one band of 311bp, HET – three bands of 311, 184, and 127bp, and MUT – two bands of 184 and 127bp.

RESULTS

5-azacytidine dose response curve

The objective of the experiment was to investigate the toxicity of 5-aza (*i.e.*, 0, 1, 3, 10, and 30 µM) in EK zebrafish. The results revealed that there was no statistical significant difference found between the treatment groups and each ELSTS time point using a two-way ANOVA ($p > 0.05$; Fig. 4). There was a statistically significant increase in the incidence of morphological abnormalities observed upon exposure to 30-µM 5-aza compared to all other experimental groups at each time point ($P > 0.05$, Fig. 4). Moreover, a statistically significant increase in the incidence of morphological abnormalities was also apparent between the 10 µM 5-aza exposure group compared to the 0, 1, and 3 µM exposure group at each time point. The frequently observed were mortality, small heads, small eyes, pericardial effusions, and tail deformities.

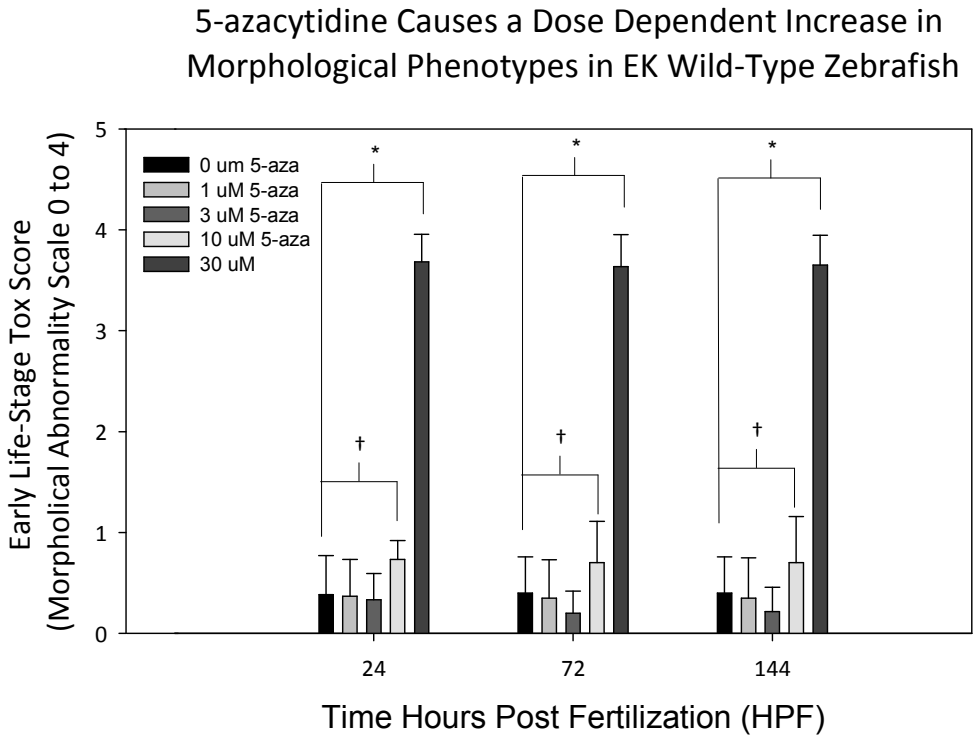

Fig. 4. 5-azacytidine causes a dose dependent increase in morphological phenotypes. There was a significant differnce ($P > 0.05$) found among all treatment by two-way ANOVA. Comparing treatments groups, there was a significant difference among the 30µM treatment and all treamtment groups. The 10 µM group also had a signficant difference among the 0, 1, and 3 µM groups. There was no differnce found among each ELSTS time points. * Indicates signficance ($P < 0.001$). † Indicates signficance ($P < 0.05$).

5-azacytidine causes DNA hypomethylation in zebrafish embryos

To evaluate whether or not we could measure DNA hypomethylation using MethylFlash™, one group of EK embryos were statically exposed to 30 µM of 5-aza for 24 hours. The second group was exposed to 30 µM of 5-aza with a fresh medium change every 8 hours. The results showed a non-statistically significant decrease in DNA methylation relative to control (Fig. 5). When a medium change was performed every 8 hour, it further decreased global methylation. One-way ANOVA of variance showed no significant difference among treatment groups ($p>0.05$).

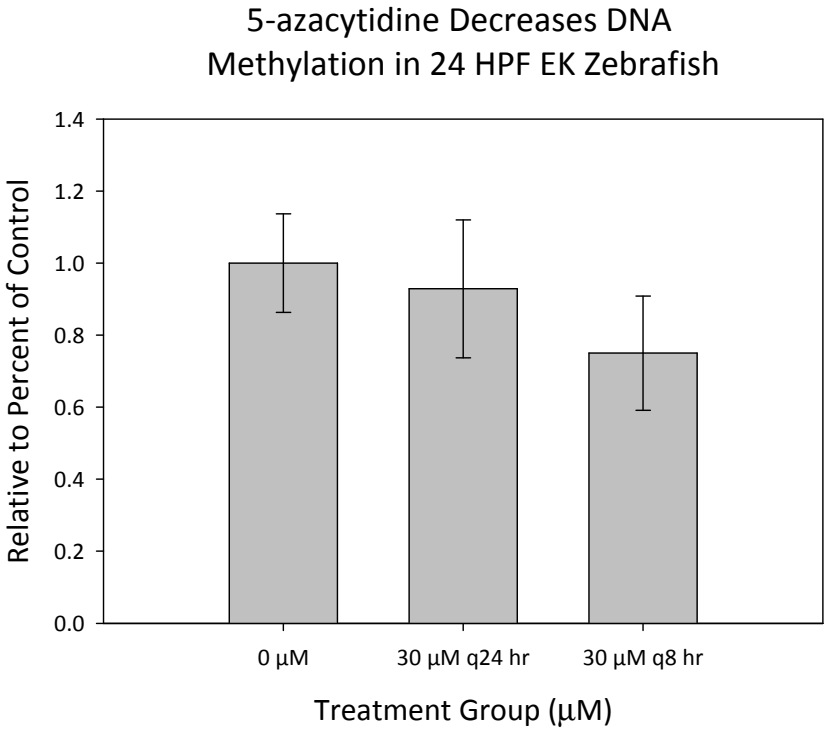

Fig. 5. 5-azacytidine causes a global decrease in DNA methylation during a static 24 hour exposure or a reoccuring 8 hour exposure for 24 hours. DNA was isolated and MethylFlash™ performed to quantify DNA methylation. Data presented has been standardized to be relative to control values with error bars as STDEV. A one-way ANOVA showed no significant difference in DNA methylation, but a trend is observed. Sample numbers are 2-4 respectively.

Methylmercury causes DNA hypomethylation in zebrafish embryos

DNA hypomethylation caused by methylmercury, was investigated by statically exposing EK embryos (1 cell stage) for 12 hours. The dose response curve (*i.e.*, 0, 0.001, 0.003, 0.01, 0.03, and 0.1 µM) was performed using MeHg. RNA free genomic DNA was isolated and MethylFlash™ was performed. There was a significant difference found among the treatment 0.03 and 0.1 µM groups using a one-way ANOVA of variance with a post-hoc Holm-Sidak multiple comparison versus control ($P < 0.05$, Fig. 6).

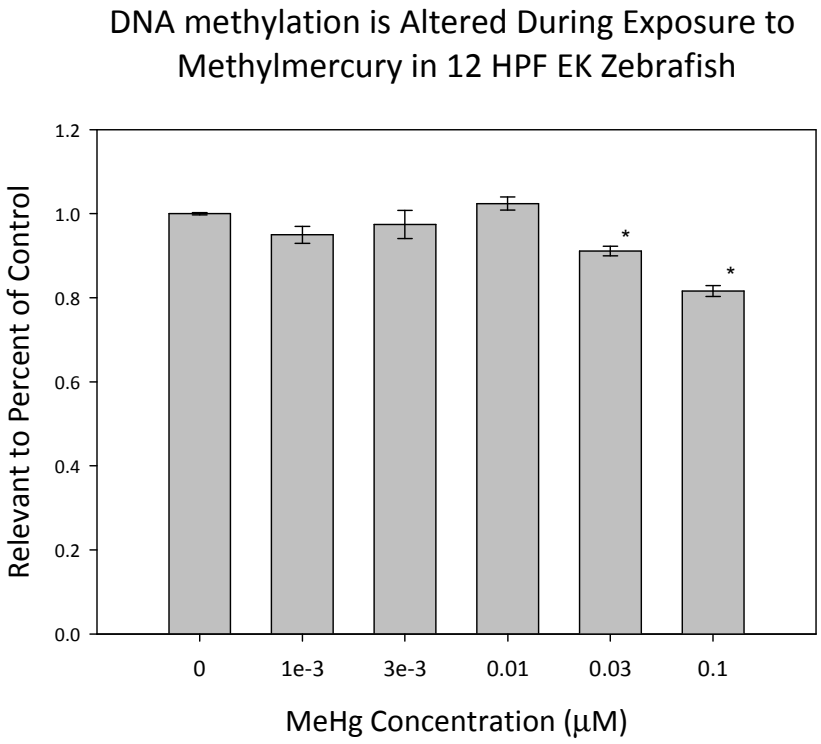

Fig. 6. DNA methylation is altered during exposure to methylmercury in 12 HPF EK zebrafish. MethylFlash^{TM} was performed to quantify global DNA methylation in pools of 50 embryos per sample with sample numbers of 3 to 7 and run in 3 seperate exposures trails. Data is presented as mean ± S.E.M. and all samples were standardized to the control on each trial day. A significant difference among the 0.03 and 0.1µM groups was found by one-way ANOVA with a Holm-Sidak Multiple Comparisons *versus* Control. * Indicates significant difference to control ($P < 0.05$).

Methylmerucry causes transgenerational DNA hypomethylation in sperm

MeHg was exposed to an F1 generation of EK embryos as previously described and raised to adults were they were bred to create an F2 and F3 generation, that had no further supplementations. Sperm was collected from the F1 and F3 generations in the 0 and 0.01 µM and MethylFlash^{TM} was performed to quantify DNA methylation and investigate any transgenerational impacts that MeHg zebrafish germ lines. There is a decrease of DNA methylation associated with methylmercury exposure seen in the data (Fig. 7). The trend was carried from the F1 generation to the F3 at almost equal levels. A student t-test was performed

to compare the 0.01 µM MeHg group to their respective generational control with no significant difference found ($P < 0.05$).

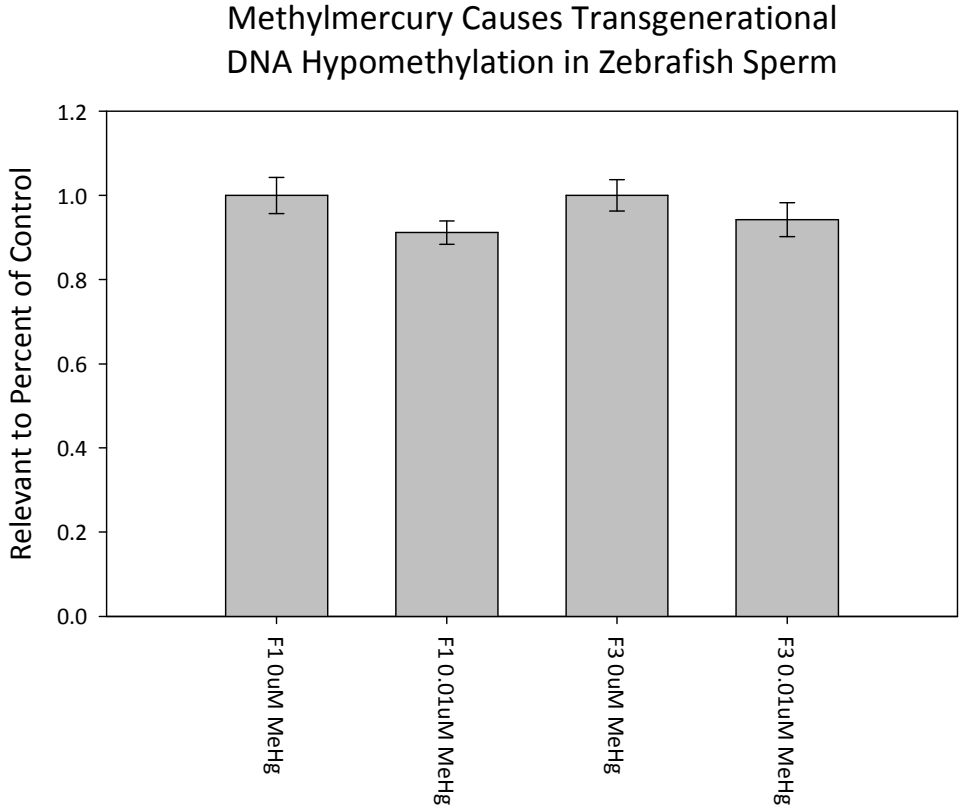

Fig. 7. Methylmercury causes transgenerational DNA hypomethylation in zebrafish sperm cells. DNA methylation was quantified in genomic DNA collected from sperm cells by MethylFlash^{TM} DNA Methylation Quantification (Colorimetric) kit. No significant difference in level of DNA methylation (t-test) between the vehicle control and the treatment group for F1 and F3, but trend suggestive of inheritable DNA hypomethylation is present. Samples were run as pools with 3 sampels run in duplicate.

5-azacytidine impairs the optomotor response and is rescued by cyanocobalamin

The optomotor response was performed on 144 HPF EK zebrafish exposed to 0 µM 5-aza, 30 µM 5-aza, 1 µM cyanocobalamin, and 30 µM 5-aza with 1 µM cyanocobalamin. There were no significant differences among treatment groups as assessed by one-way ANOVA; however, there was a drastic decrease in the number of animals that passed the test in the 30 µM 5-aza treatment group (Fig. 8). A t-test analysis comparing the 0 µM 5-aza and the 30 µM

5-aza resulted ina P value of 0.065, suggesting that these data may become significant with an increased number of animals. There was no impairment of the OMR in the 1 µM cyanocobalamin. Co-exposure of 1 µM cyanocobalamin with 5-aza appears have increased the amount of animals that passed the test to that of the control level.

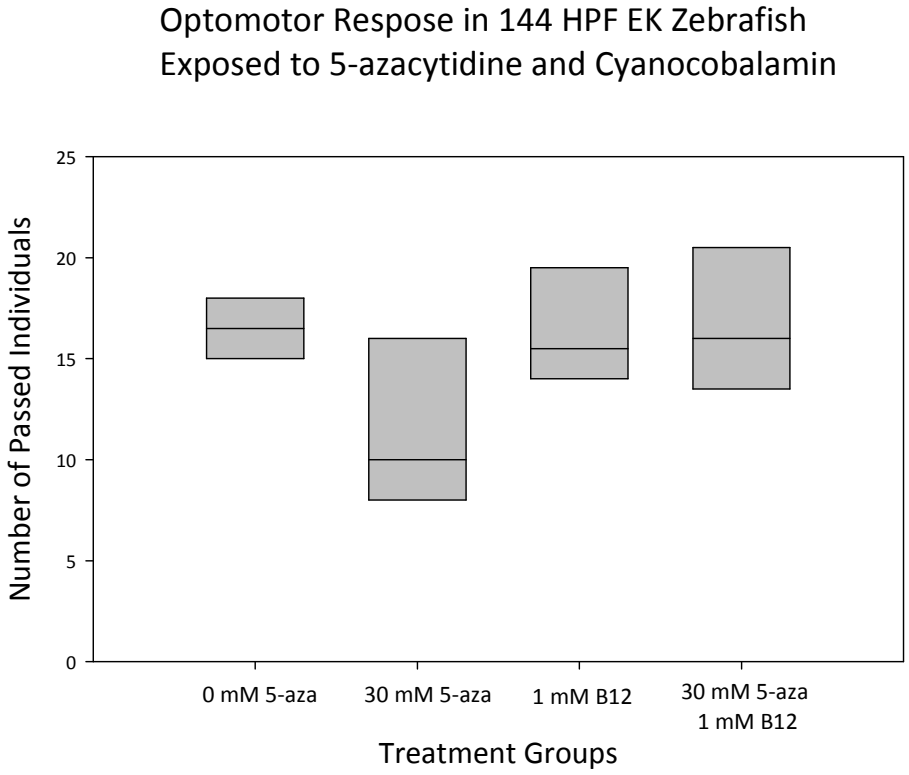

Fig. 8. Co-exposure of methylcobalamin recovers alterations in DNA hypomethylation induced optomotor response in 144 HPF EK zebrafish. Exposure to 30 µM 5-aza caused a decrease in number of passing animals. 1 µM methylcobalamin exposure shows no significant change in comparison of the control. Co-exposure of both 5-aza and cyanocobalamin shows a recovery of the 5-aza phenotype to the level of the control. Statisitical evaulation using a student t-test between the control group and 30 µM 5-aza group had a P value = 0.065.

C-start response is not grossly altered in dnmt1 s^{872} at 72 HPF

To investigate the effect of global DNA hypomethylation on neurological synaptic transmission and plasticity, a touch response assay was performed at 72 HPF. This was performed using the *dnmt1* S^{872} zebrafish to investigate the effects of hypomethylation in the absence of a toxin. A touch near the otolith in the WT *dnmt1* S^{872} elicited a rapid escape from the stimulus which can be clearly observed without the use of high speed imaging. Touch stimulation of heterozygous and mutant fish also resulted in rapid escapes (Table 1). No significant differences were found among groups using a Kruskal-Wallis one-way ANOVA of variance of ranks.

Table 1. C-start response is not altered in *dnmt1* S^{872}

Zygosity	Touch Response Score	STDEV	Sample #
Wild-Type	1.95	±1.95	38
Heterozygote	1.94	±1.94	131
Mutant	1.89	±1.89	52

Circadian rhythm is altered in dnmt1 s^{872} mutant zebrafish

In this study, we tested the effects of DNA hypomethylation on the biological clock by evaluating circadian rhythm. Time points used in the analysis corresponded to the scheduled lights on/off (9:00/23:00) times; as well as, two hours before and after the trained light changes. No statistically significant differences were found between the wild-type and heterozygous groups (Fig. 9-11). There were not enough mutants available in each individual

trial to perform statistical analysis. Trials were first analyzed alone in order to show trial-to-trail variations in zebrafish activity. Two-way ANOVA was performed comparing genotypes in each one-hour time bin for each individual trial.

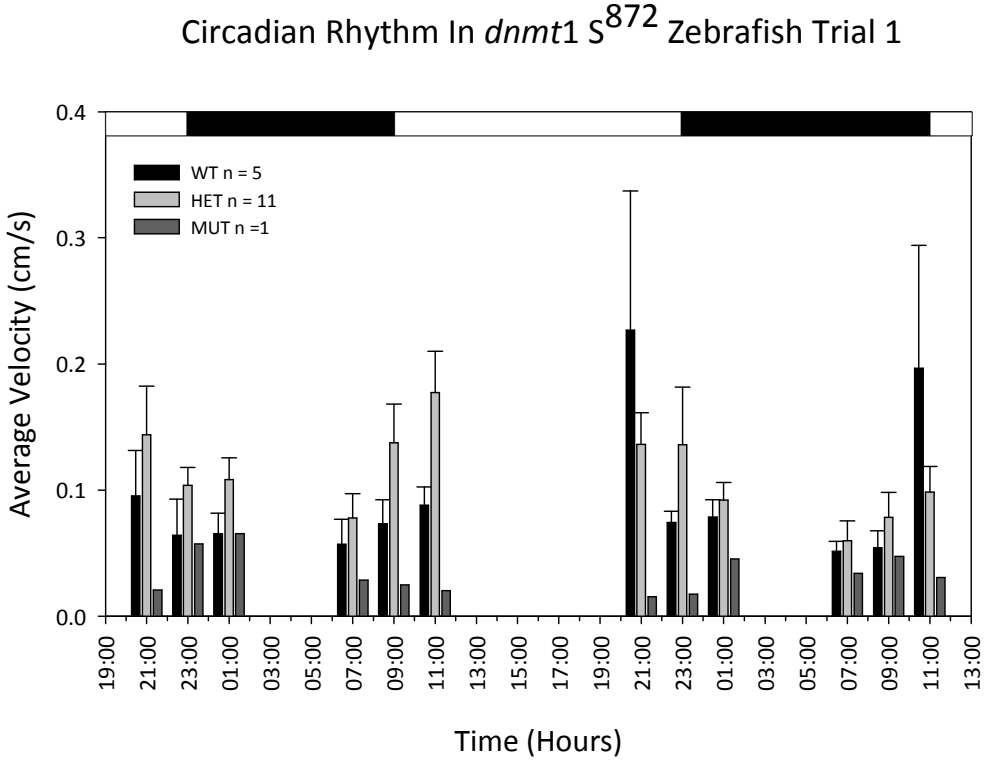

Fig. 9. Ciracdian rhythm is not altered in *dnmt*1 S^{872} zebrafish in Trial 1. Circadian rhythm was performed at 5 DPF and a recording at a constant low level of light was done for 56 hours. The light phase bar is shown to indicate the normal ligth cycle the larvae were raised in. Data is presented in 1 hour time bins and time points presented are when normal light on/off (9:00/23:00) and 2 hours before and after those periods. No significant differences were found between groups by two-way ANOVA.

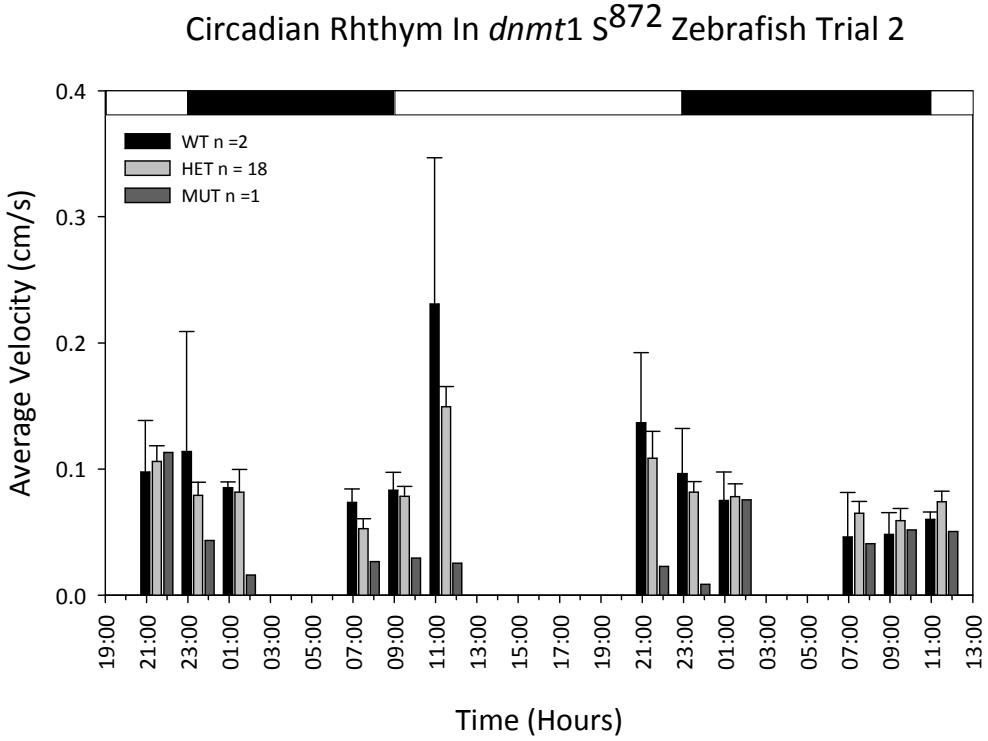

Fig. 10. Ciracdian rhythm is not altered in *dnmt*1 S^{872} zebrafish in Trial 2. Circadian rhythm was performed at 5 DPF and a recording at a constant low level of light was done for 56 hours. Data is presented in 1 hour time bins and time points presented are when normal light on/off (9:00/23:00) and 2 hours before and after those periods. No significant differences were found between groups by two-way ANOVA.

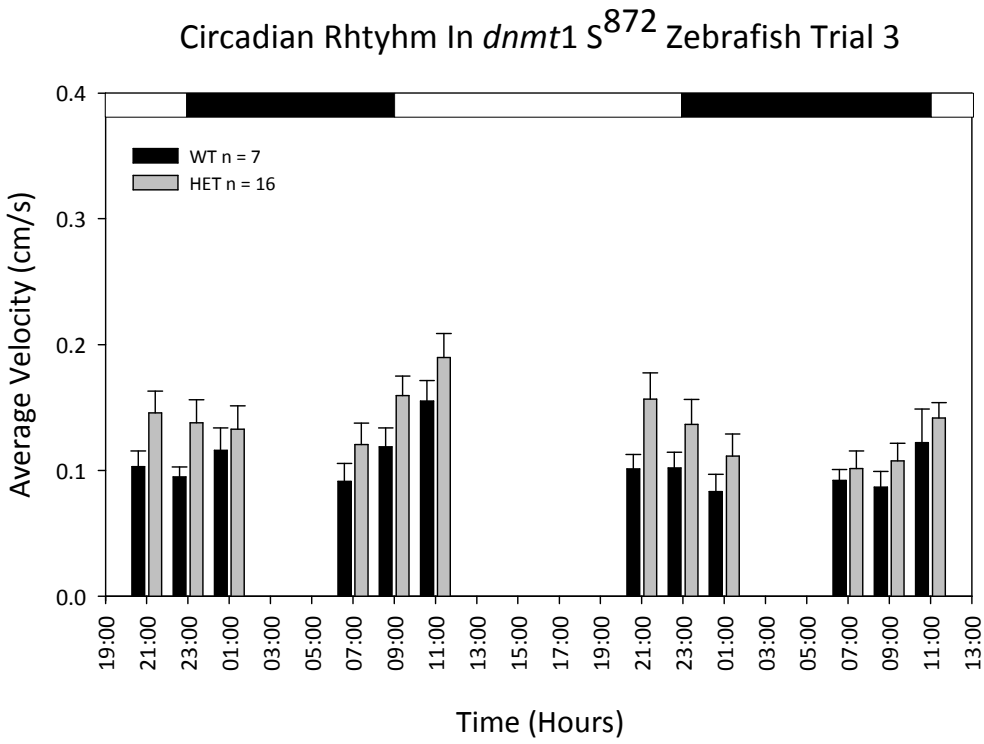

Fig. 11. Ciracdian rhythm is not altered in *dnmt*1 S^{872} zebrafish in Trial 3. Circadian rhythm was performed at 5 DPF and a recording at a constant low level of light was done for 56 hours. The light phase bar is shown to indicate the normal ligth cycle the larvae were raised in. Data is presented in 1 hour time bins and time points presented are when normal light on/off (9:00/23:00) and 2 hours before and after those periods. No significant differences were found between groups by two-way ANOVA.

With no significant findings in the individual circadian rhythm trials, the three trials were pooled together. Although this provided some statistical power for comparison of the mutants; the sample size was still too low. There were no statistically significant differences found between any groups using a two-way ANOVA (Fig. 12). However, using Kruskal-Wallis one-way ANOVA of variance of ranks on each time period there was a significant ($P < 0.05$) difference between the heterozygous and the mutant groups at the six DPF, 11:00AM time point. No other time points were significant.

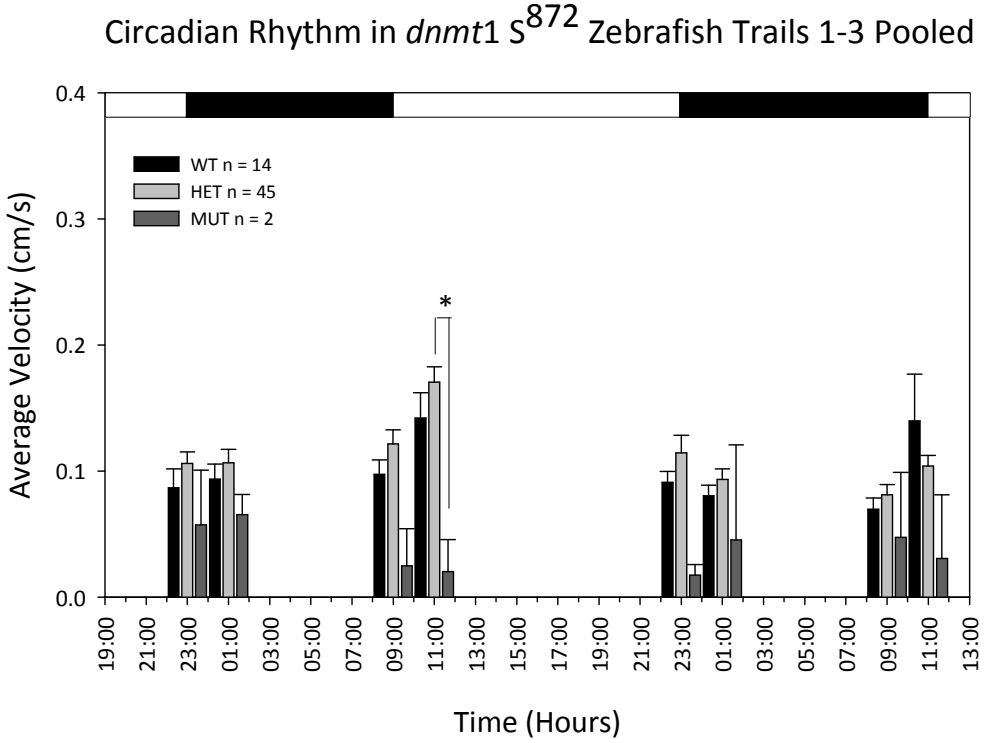

Fig. 12. Ciracdian rhythm is not altered in *dnmt*1 S^{872} zebrafish in Pooled Trials. Circadian rhythm was performed at 5 DPF and a recording at a constant low level of light was done for 56 hours. The light phase bar is shown to indicate the normal ligth cycle the eleutheroembryos were raised in. Data is presented in 1 hour time bins and time points presented are when normal light on/off (9:00/23:00) and 2 hours after those periods. No significant differnece was found by two-way ANOVA. A significant difference was found by one-way ANOVA between the heterzygous and mutant group at 11:00 hour of the 6 DPF. * Significant difference ($P < 0.05$).

Locomotion is not altered in the dnmt1 S^{872} zebrafish

Locomotion was tested by evaluating activity in different light phases. The first trial was in a session of 30 minutes of constant darkness. (Fig. 13 A). The *dnmt*1 S^{872} zebrafish exhibited low levels of activity in all groups. There were no significant differences found by two-way ANOVA with repeated measures.

The second trial for locomotion was a 30-minute session of white light. The *dnmt*1 S^{872} zebrafish showed an initial low locomotor activity with a steady increase as the trial continued (Fig. 13 B). There were no significant differences by two-way ANOVA with repeated measures.

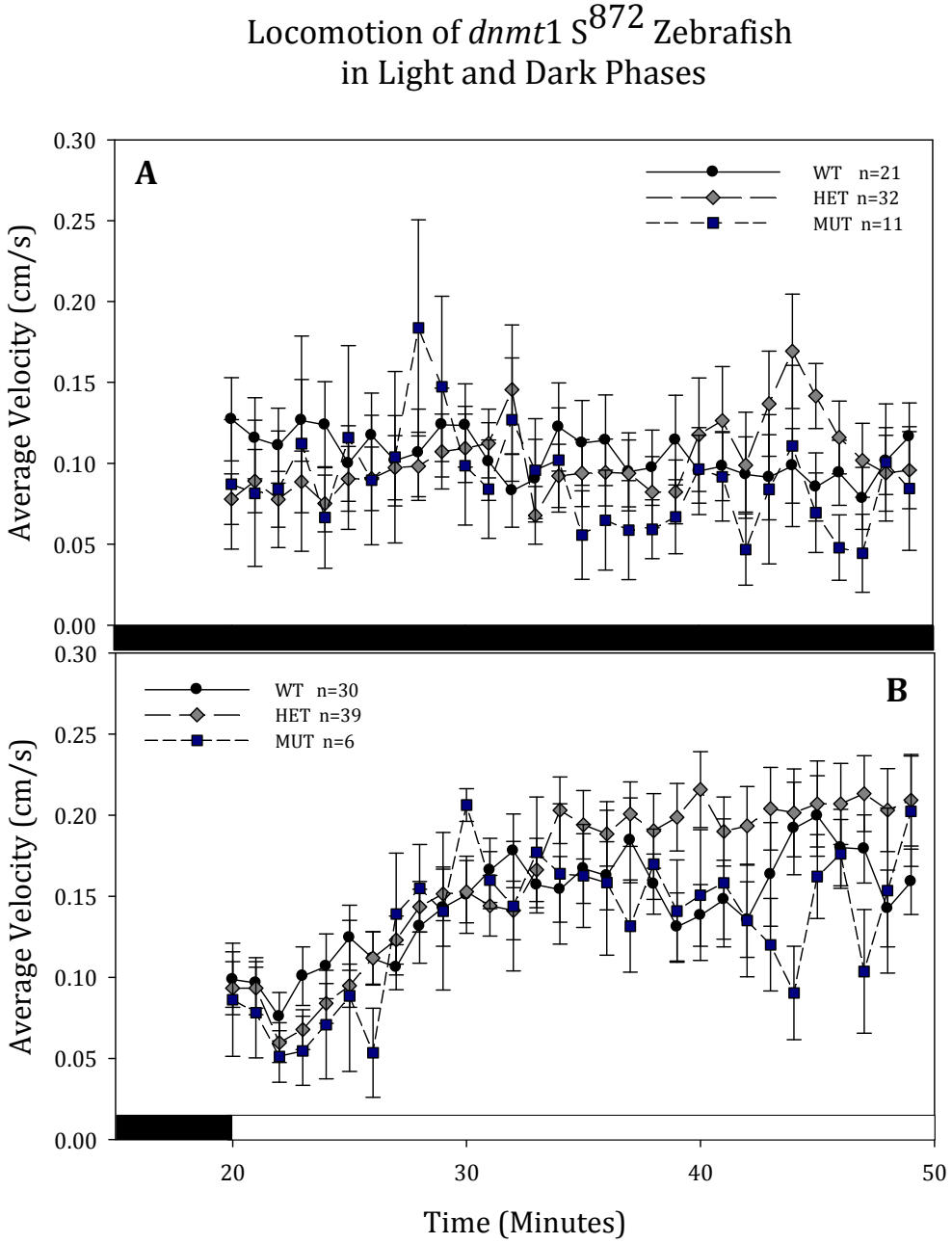

Fig. 13. Locomotion of *dnmt*1 S^{872} Zebrafish in not altered during Light and Dark Phases. Light status is indicated by colored bars during each session. **A**. Locomotion in 30 minutes of darkness resulted in low locomotor activity in all genotypes without any significant differences. **B**. Locomotion in a 30 minute of light resulted in an initial low activity, but steadily increased over time. There was no significant differences. Both trails were analyzed by two-way ANOVA with repeated measures.

Visual-motor response is altered in the dnmt1 S^{872} mutants

The VMR assay uses alternating ten-minute intervals of light and dark periods to assess the behavioral response of the fish to sudden changes in light. Although each genotype responded to the light-phase changes, there was a decrease in locomotor activity as global DNA methylation decreased (Fig. 14). Averaging each ten-minute phase change and performing a Kurskal-Wallis one-way ANOVA of variance of ranks in each phase showed a significant difference between the wild-type and the mutant strains at the first and third dark phases (Fig. 15). There were no statistically significant differences found between the heterozygous and the wild-type groups.

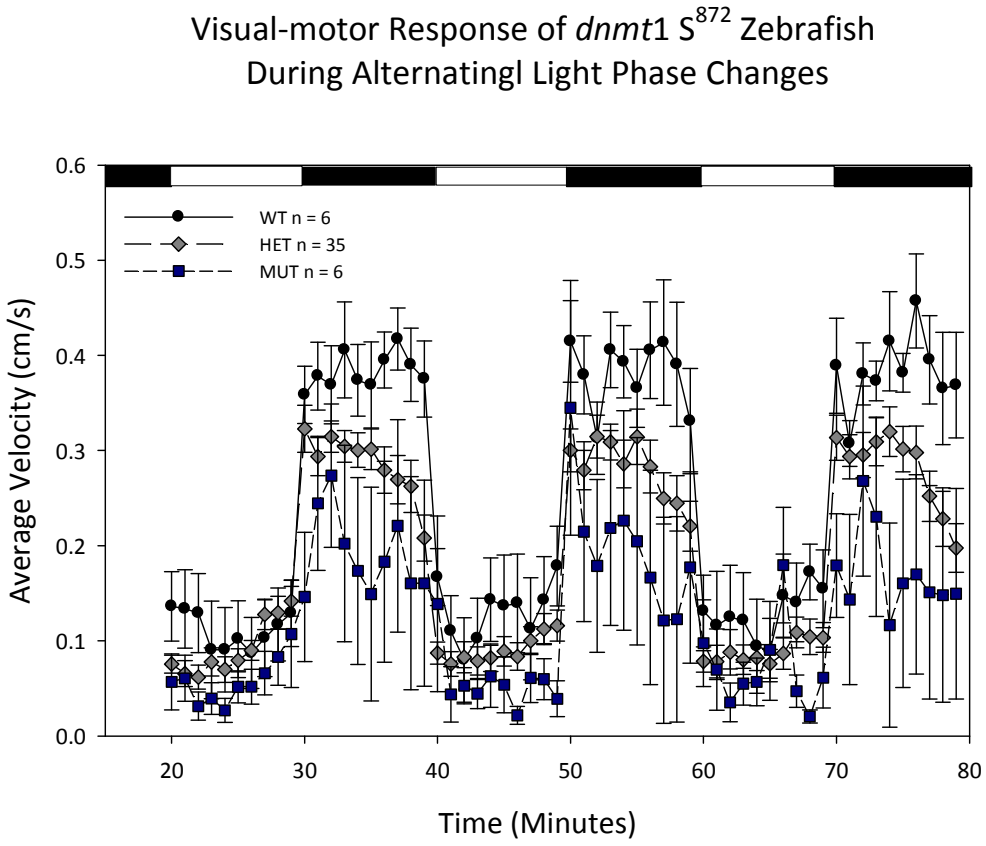

Figure 14: VMR activity is altered in *dnmt*1 S^{872} during alternating light phases. During the light phases, a decrease in locomotor activity was observed, while a increase in locomotor activty when the lights were shut off. There is a gradient decrease in locomotor activty observed between all groups, with the wild-type having the greatest level of activity and the mutatns have the lowest amount of activity. There was a significant distance found between zygosity and time using a two-way ANOVA.

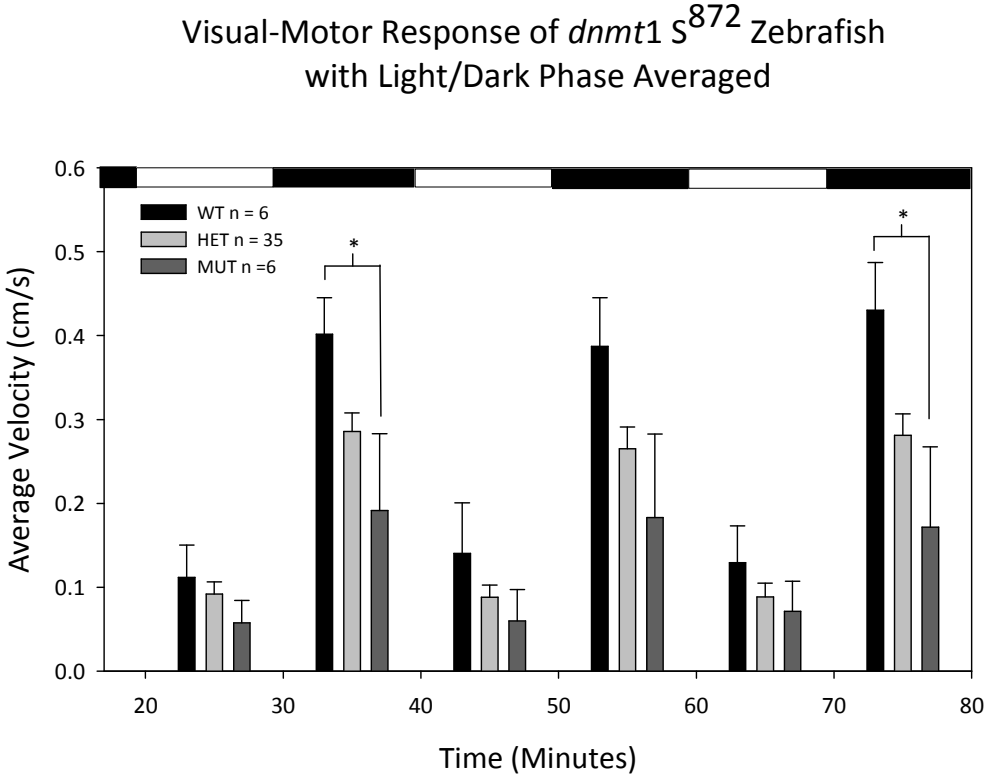

Figure 15. VMR is altered in *dnmt*1 S^{872} mutants. Averaging each 10 minute phase together and comparing locomotor activity showed a significant decrease in locomotor activity in mutant groups during the first and thrid dark phases by one-way ANOVA ($P < 0.05$). A significant difference was not found in the light phases or bewteen the heterzygous groups in any phase. The light phase bar is shown to indicate the phase changes. * Signficant difference ($P < 0.05$).

C-start response is not grossly altered in dnmt1 s^{872} at 144 HPF

A second touch response was performed at 144 HPF after a locomotion trial to investigate further development of the neurological system at a later time period. All individuals had a quick and rapid response (data not shown).

DISCUSSION

Methylmercury (MeHg) is an environmental toxicant that presents a high concern for public health, as many people around the world ingest organic mercury through dietary fish consumption (Grandejean *et al*., 2010; Newland et al., 2008). For instance, Tsankova *et al*. (2006), discovered that MeHg induced long-lasting developmental problems in perinatal exposed mice. Specifically, the results showed DNA hypemethylation, increased H3-K27 tri-methylation, and decreased acetylation on the promoter site IV in brain-derived neurotrophic factor (*Bdnf*), an important protein that helps with nerve growth, function and survival. Their findings also support that sub-lethal environmental toxins could mediate epigenetic modifications in neurons that, in turn, lead to long-term neurobehavioral phenotypes Moreover, Bose *et al*. (2012) reported that globally decreased DNA methylation was inherited in NSC daughter cells after a low dose of mercury in the parental cell. The epigenetic mechanisms whereby different toxicants influence global gene expression changes that, ultimately, yield abnormal neurobehavioral phenotypes remain unknown.

The objective of this study was to investigate the impact of DNA hypomethylation the neurobehavior of zebrafish larvae. First, we exploited 5-azacytidine (5-aza) and MeHg, established demethylating agents (Pilsner*et al*. 2009; Bose *et al*., 2012; Oakes *et al*., 2007), to investigate the impacts of epigenetic changes on neurobehavior. We also employed the *dnmt*1 S^{872} mutant zebrafish to characterize potentially altered neurobehavioral phenotypes induced by global DNA hypomethylation in the absence of toxicant. If similar phenotypes are found in the absence and presence of an environmental toxicant, then possible mechanistic outcomes in neurobehavior abnormalities can be associated with dysregulated DNA methylation.

5-aza was used to chemically induce global DNA hypomethylation in zebrafish during the first 24 hours of development. 5-aza binds to the catalytic domains of DMNTs and inhibits their function (Santi *et al*., 1983). The magnitude of global DNA methylation was assessed using MethylFlash™ analysis following exposure to 30-µM 5-aza, which was the highest dose tested that produced some morphologically normal embryos. Although the sample size was not large enough to identify a significant difference, 24-hour exposure to 30 µM 5-aza exhibited a downward trend in the level of DNA methylation that necessitates further research (Fig. 5). Clinical trials have shown that 5-aza has a half-life of 4 hours and is completely undetectable after 8 hours (Rudek*et al*., 2005). That said, when embryos were exposed to fresh 5-aza every 8 hours for 24 hours, DNA hypomethylation was much more apparent as a result of methylation being continuously inhibited (Fig. 5). The variation with these data could stem from the potential of its quick half-life, thus creating different dose zone across the plate, where animals were exposed to different levels of 5-aza. Since the chosen animals were morphologically normal, it is also possible that the data variation is a result of variable tolerance to the effects of 5-aza. Ultimately, further experimentation is required to validate that results described above.

Having determined that 5-aza can induce DNA hypomethylation in developing zebrafish, this chemical was exploited to investigate the effect of toxicant-induced DNA hypomethylation on optomotor response (OMR). The OMR is an innate behavior in zebrafish and starts at 5 DPF (Portugues and Engert, 2009). It involves the proper function of the retinal ganglion cells, rods and cones, and neurocircuits (Roeser and Baier, 2005; Orger and Baier, 2005; Orger*et al*. 2008). Results showed that 5-aza caused a decrease in the number of animals with a functional OMR (Fig. 6). Next, the hypothesis proposed by Lee *et al*. (2009) was investigated using 5-aza. Lee *et*

al. (2009) hypothesized that the presence of an environmental toxicant can shift the methionine pathway towards GSH synthesis resulting in decreased methionine synthesis (and reduced DNA methylation) but could be restored by methyl donor supplementation. Therefore, zebrafish embryos were exposed to 30-µM 5-aza supplemented with 1-µM cyanocobalamin (a well-characterized methyl donor). Cyanocobalamin alone did not cause any significant difference in the OMR, but the co-exposure of 5-aza and cyanocobalamin increased the number of animals from an average of 40% passed in the 5-aza alone to 64% in the co-exposure, which was close to the level of the control fish (Fig. 8). These data support the hypothesis proposed by Lee *et al*. (2009), suggesting that the addition of the methyl donor, cyanocobalamin, increased the level of methionine synthesis as GSHis an antioxidant that scavenges free radicals, plays a key role in detoxification and helps prevent apoptosis (Leggatt and Iwama, 2009; Deth*et al*. 2008). GSH is known to bind to MeHg to help eliminate it out of the organisms, which leads to a drastic depletion of glutathione and increased risk of cellular damage (Sanfelie et al., 2001; Yee and Choi, 1996).The next objective of this study was to investigate and compare the effects of methylmercury to those of 5-aza.The acute effects of methylmercury toxicity in humans are neurological toxicity, multiple organ toxicity, immune system disorders, and cellular oxidative stress (Li *et al*., 2010; Mingwei *et al*., 2010). Low dose exposures of MeHg are also of concern as even in woman that are not symptomatic (Grandjean and Herz, 2011), it has been shown that perinatal exposure in rats causes neurobehavioral impairments later after birth (Fujimura *et al*., 2012).

The results of the current study support the notion that MeHg can cause DNA hypomethylation as seen in previous investigations (Pilsner *et al*. 2009; Bose *et al*., 2012; Oakes

et al., 2007), and that the MethylFlash™ assay is an effective means of quantifying DNA methylation in zebrafish. To further assess epigenetic changes from MeHg exposures, global DNA methylation in sperm was investigated. Sperm is a germ-line cell with an imprinted methylation pattern that is passed to the offspring during conception and is a important factor in genetic inheritance (Jirtle and Skinner, 2007). EK embryos (1-cell stage) were exposed to MeHg for 24 hours to generate a F1 generation. These embryos were then raised to adults with no further exposures to MeHg and were bred to make the F2 and F3 generations. Sperm was then collected from F1 and F3 males (0 µM and 0.01 µM exposure groups only) and pooled into groups of three for MethylFlash™ analysis. There was a downward trend in the DNA methylation of both generations, but a statistically significant difference was not observed due to small sample size (Fig. 7). However, it is important to reiterate that a reduced level of global DNA methylation in the F3 generation was apparent despite the lack of direct exposure to MeHg. Thus, epigenetic conservation is solely by germ-line transgenerational inheritance. This report analogous to that of Guerrero-Bosagna and Skinner (2012) regarding germ-line inheritance of epigenetic patterns. In addition, Oakes et al. (2007) reported DNA hypomethylation and impaired function of sperm in *Dnmt1+/-* mice exposed to 5-aza-2-deoxycytidine (a DNMT inhibitor). If both MeHg and 5-aza-2-deoxycytidine cause DNA hypomethylation, then it is plausible that they share similar mechanisms. Methylmercury has been shown to increase GSH levels during exposure; however, MeHg-induced disruption of GSH homeostasis is not the only means of toxicity (Ou *et al*., 1999; Fujimura *et al*. 2012; Grandjean and Herz, 2011; Ginsberg *et al*., 2014). We suggest that an additional MeHg-induced mechanism of toxicity is DNA methylation changes caused by decreased availability of

methionine.The mechanism shared by MeHg and 5-aza seems to be strictly related to DMNT function as both chemicals have been shown to interfere with their function (Basu *et al*., 2013; Santi *et al*., 1983). This hypothesis can be evaluated further using the *dnmt*1 S^{872} mutant zebrafish and investigating neurobehavioral outcomes.

The next research objective was to evaluate the effect of hypomethylation on neurobehavior using *dnmt*1 S^{872} zebrafish. This C-start assay is used to evaluate early neurological plasticity and synaptic transmission, particularly in the Mauthner cells, the largest neurons in the zebrafish central nervous system (Kimmel *et al*., 1981). There was no gross significant difference in the C-start response in and of the *dnmt*1 S^{872} eleutheroembryos (Table 1.) Conservation of this circuit shows that early neurodevelopmental impacts are not grossly inhibited by the *dnmt*1 S^{872}-induced global hypomethylation. To exclude a problem in later neuronal development leading to a dysfunction in motor neuron function, a second C-start touch response assay was performed on 144 HPF *dnmt*1 S^{872} zebrafish. Again all animals escaped effectively. This provides further evidence of potential neurologic abnormalities in the brain *versus* a sensory and motor neuron abnormality; as the zebrafish maintained their sensory motor neurons and rapid-escape responses. It should be noted that high-speed imaging and analysis of tail beat frequency and degree of bend were not conducted in this experiment. There may therefore, be a subtle decrease in response efficiency that was not investigated and may be present.

The circadian rhythm was slightly altered in *dnmt*1 S^{872} heterozygous animals and almost completely abolished in the *dnmt*1 S^{872} homozygous animals. Circadian rhythm is an important biological clock that is controlled by the suprachiasmatic nucleus in the brain

(Rosenwasser*et al*., 2014) and which controls daily functions and energy levels. Although this study does not possess sufficient statistical power due to small sample sizes, the heterozygous individuals show a trend toward hyperactivity that is associated with DNA hypomethylation. There have been multiple studies that have looked at the level of specific gene expression in zebrafish that have neurobehavioral alterations in response to DNA hypomethylation. Parrish *et al*. (2013) found that *DNMT* inhibition (by zebularine exposure) in epileptic rats decreased field excitatory postsynaptic potentials in the hippocampaus; as well as causing an increase in a glutamate receptor subunit epsilon-2 (Grin2b/Nr2b) expression. The excitatory post synaptic potential in neurons are important for making the neuron more likely to fire an action potential (Knogler and Drapeau, 2014).If DNA hypomethylation alone can alter the excitatory post snynaptic potential, then neural latency of the action potential could arise. Similar results were reported by Parrish *et al*. (2013) as they suggested that DNA hypomethylation also causes posttranscriptional mechanistic changes in neurons as it promoted a latency phase in the onset of seizures in their model. In terms of the current study, the *dnmt*1 S^{872} mutants, with severe DNA hypomethylation, may have contributed to the decrease in the activity throughout the circadian rhythm trial by slowing down the action potential depolarization. The alterations in circadian seen in this study may also be contributed to hormonal changes. In animals that have seizures with or without convulsions, it is theorized that behavioral phenotypes can still arise from stress-induced cortisol increases that influences seizure-related behaviors and endocrine phenotypes (*i.e*., hyperactivity; Wong *et al*., 2010). Another interesting finding is the fact that circadian rhythm in not completely regulated by the SCN in zebrafish like it is in mammals (Noche et al., 2011). Whitmore *et al*., (1998, 2000) performed studies looking at circadian

rhythm in zebrafish that completely lacked SCNs and found that their mutant *cyc* fish, still had a functional rhythm. They isolated organs and in cell culture were able to show that a separate oscillator were present in kidney and heart in zebrafish and were still entrained by light and dark cycles. In the *dnmt*1 S^{872} fish there is drastic onset of apoptosis of hepatic and pancreas cells around 8 DPF (Anderson *et al*., 2009). If circadian rhythm supporting oscillators exist in multiple organs within zebrafish, then the almost complete abolishment of the circadian rhythm in the *dnmt*1 S^{872} mutants (Fig. 12) may be a result of the altered physiology exhibited by these organs. In general, these mutants may not be a good candidate for circadian rhythm trials past 7 DPF.

With problems in circadian rhythm apparent, the locomotion and VMR trials were performed to investigate motor and visual-motor response. Locomotion was consistent across all genotypes of *dnmt*1 S^{872}zebrafish when assessed in 30 minutes of constant darkness or light (Fig. 13A-B). The quicker 10-minute intervals of light and dark periods of the VMR assay resulted in decreased average velocity during dark phases in relation to the severity of DNA hypomethylation (Fig. 15). These data suggest that the *dnmt*1 S^{872} mutant zebrafish can visually see the light changes; however, they seem to have a problem in appropriately responding to the stimulus. The effects of light are mediated by a non-image-forming visual pathway that starts in the eye by melanopsin-expressing intrinsically photoreceptive retinal ganglion cells (Hattar *et al*., 2003). It is assumed that if these retinal ganglion cells are defective due to altered gene-expression, there would be a delayed response to the light-period change; however, in our study the fish responded immediately and consistently to each phase change while the magnitude of response was altered. MacPhail et al. (2009) proposed that the changes

in VMR could be from alterations in paradoxical activity, where dark periods create a more rapid-acting excitatory effect. When the zebrafish are given a quick alternating light-dark phase, a rebound excitation in the dark period develops. This suggests that during the changes in the light, the dnmt1 S^{872} heterozygous and mutant groups may have a dysfunction in neural excitation. In relation to humans, problems involved in neural excitations can be seen in kinesia, which is controlled by the basal ganglia and dopamine receptors as investigated by Sano *et al*., (2013). There is also supporting information by Irons et al. 2012 that emphasize sthe importance of dopamine receptors in motor behavior. In the future, investigating dopamine receptors using agonists and antagonists may elucidate a possible neural mechanism of zebrafish that could be altered from DNA methylation.

In summary, we have shown that the *dnmt*1 S^{872} strain of zebrafish is a good candidate model for investigating the effects of DNA hypomethylation on early neurobehavioral outcomes. The heterozygous and the mutant *dnmt*1 S^{872} zebrafish provided a global reduction in DNA methylation without the direct cytotoxicity by environmental toxicants or other DNA hypomethylating agents. The neurobehavior phenotypes found in this study can be strongly associated with DNA methylation changes; however, secondary synergistic mechanisms require further research.. In this study, we were able to show that circadian rhythm and locomotor activity are potentially regulated via DNA methylation; however, more samples are needed for appropriate statistical power. The *dnmt*1 S^{872} fish responded to touch stimulus and had no apparent morphological phenotypes, thus functional neural transmission was conserved. Interestingly, a DNA hypomethylation-induced OMR phenotype may have been manipulated by co-exposing zebrafish embryos with cyanocobalamin and 5-aza; probing the theory that the

methionine pathway is a key contributor epigenetic modulation during environmental toxicant exposures. Collectively, the knowledge gained here and future, investigation into the specific role of individual neuronal cell types, neurocircuits, and molecular neurotransmitters will further elucidate the mechanism in which DNA methylation regulates neurobehavior.

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