



Zebrafish as an integrative vertebrate model to identify miRNA mechanisms regulating toxicity



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ABSTRACT

Zebrafish (*Danio rerio*) are an integrative vertebrate model ideal for toxicity studies. The zebrafish genome is sequenced with detailed characterization of all life stages. With their genetic similarity to humans, zebrafish models are established to study biological processes including development and disease mechanisms for translation to human health. The zebrafish genome, similar to other eukaryotic organisms, contains microRNAs (miRNAs) which function along with other epigenetic mechanisms to regulate gene expression. Studies have now established that exposure to toxins and xenobiotics can change miRNA expression profiles resulting in various physiological and behavioral alterations. In this review, we cover the intersection of miRNA alterations from toxin or xenobiotic exposure with a focus on studies using the zebrafish model system to identify miRNA mechanisms regulating toxicity. Studies to date have addressed exposures to toxins, particulate matter and nanoparticles, various environmental contaminants including pesticides, ethanol, and pharmaceuticals. Current limitations of the completed studies and future directions for this research area are discussed.

1. Introduction

Zebrafish (*Danio rerio*) are an established model for developmental biology due to their physiology, genetics, and assaying potential. Embryos develop *ex vivo*, offering a non-invasive approach to study developmental events. Transparency of embryos and larvae in early developmental stages make it easy to visualize internal organs/structures and embryogenesis is complete in only 3 days (Fig. 1). In the first few hours after fertilization, rapid cell division occurs in the zygote, cleavage, blastula, and gastrula periods. Between 24–48 hours post fertilization (hpf) the brain has 5 lobes, the embryo increases rapidly in length, the circulatory system forms, and the heart begins to beat. Between 48–72 hpf, the craniofacial cartilage development allows the mouth to become distinct and rudimentary gills develop. By 72 hpf, most of morphogenesis is completed [1]. From 72–120 hpf, zebrafish are referred to as elutheroembryos. While in the process of hatching the embryo is not very active, but the elutheroembryo stage is noted with gradual increased activity (swimming, jaw, eye, and fin movement). At 96–120 hpf it is common for behavioral assays for light/dark, acoustic stimuli, and toxicological responses to be initiated [2]. Behavioral assays, such as the photomotor response can be used to screen thousands of neuroactive drugs [2–4]. In addition, zebrafish have a fully sequenced genome with the capability of creating genetic

models using multiple gene editing tools including CRISPR-Cas9 technology, making them ideal for mechanistic and discovery based studies on how genes impact physiology and behavior [5,6]. Due to assaying potential and genetic strengths, zebrafish are a good model for human translation, because zebrafish have 70 % gene homology which increases to 82 % when considering genes related to human disease [7]. Additionally, there is high conservation of metabolizing enzymes [3,8].

As noted above, the zebrafish model organism can be used in high-throughput assays for drug discovery and chemical toxicity screening [3,9–11]. Due to their developmental and biomedical strengths zebrafish are increasingly being used to define mechanisms of toxicity [2,12,13] with recent reviews highlighting the strengths of the zebrafish as a model for toxicology [3,14]. Taking advantage of the developmental and biomedical strengths, the US FDA has used zebrafish toxicity assays for drug approval [3,15]. Many toxicological studies are also evaluating chemical effects on a protein, organ, and organismal level to provide a multi-level approach to understanding how a chemical affects health at different life stages and then determining human translation relevance [16]. Since zebrafish have shorter life periods, there is also the advantage of looking at the effects of a developmental exposure throughout the lifespan in the developmental origins of health and disease (DOHaD) paradigm, and even across generations. These advantages have made the zebrafish the most common fish model to

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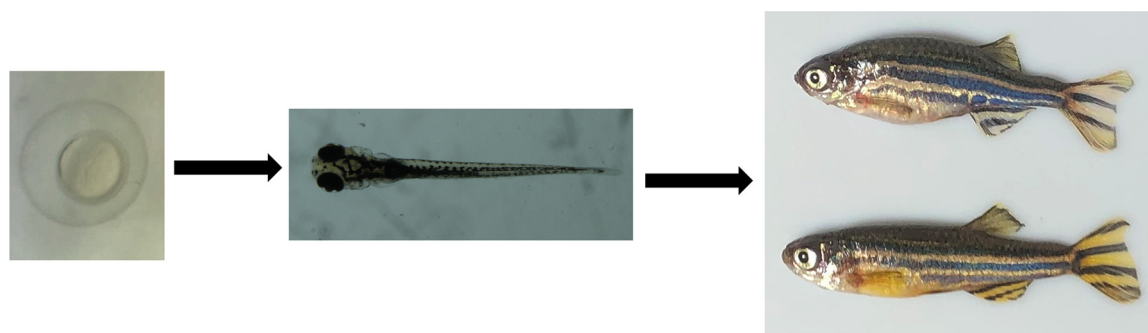


Fig. 1. Zebrafish life cycle. Images are of an embryo at 1 h post fertilization (hpf), an eleutheroembryo at 5 days post fertilization (dpf), and adult female (top) and male (bottom) zebrafish.

perform multi- and transgenerational studies evaluating epigenetic mechanisms of toxicity [14].

MicroRNAs, also known as miRNAs, are one of three currently known epigenetic mechanisms that help regulate gene expression, alongside methylation and histone modifications, without altering DNA sequence [17,18]. miRNAs are single strand noncoding RNAs that are about 22 nucleotides in length that can regulate gene expression during and after transcription to form proteins [19–21]. miRNAs are highly concerted in a time and tissue specific manner, and are important for gene regulation during development [12,22,23]. miRNAs work through a cascade of molecular mechanisms. miRNAs are transcribed by RNA polymerase II to generate primary miRNA, which are then cleaved by RNase III enzyme in the nucleus. This cleaved molecule, known as the precursor miRNA is further cleaved by Dicer to form a mature miRNA [24,25]. To inhibit genes, miRNAs arrange into a multi-protein complex to form an RNA-induced silencing complex. This complex then binds to the 3' UTR ends of the target mRNA of interest to silence the gene [24,26]. miRNAs are important because they help regulate life cycles in eukaryotic cells, molecular and metabolic processes, immunity, and stress response [21]. miRNAs are also important in pathogenesis and genetic disorders. The presence of miRNAs extracellularly was first described in 2008 in a lymphoma patient’s plasma [27]. Research with the zebrafish has also implicated the importance of miRNAs in several biological and disease pathways including heart formation, function, and disease; cancer signaling pathways; and reproduction [18,28–31]; [23]; [32]. miRNAs can be altered with disease state or xenobiotic exposure, secreted extracellularly, and detected in biofluids in humans. As such, miRNAs have become a popular biomarker to help predict risk to xenobiotic exposure.

Although many miRNAs are now identified in humans and multiple animal models including the zebrafish (Fig. 2), mechanisms of how xenobiotic exposure affects miRNA regulation is unclear, though several ideas have been proposed. For example, DNA damage can cause p53 and miRNA to work together in the nucleus to repair damage [21].

Nucleophilic sites of miRNA precursors can be bound by electrophilic metabolites, forming adducts, and preventing their ability to be worked on by Dicer in the cytoplasm. miRNAs can be absorbed by long non-coding RNAs (lncRNAs), which can then lead to altered translation and over expression of target genes [21]. In this review, we highlight the intersection of how toxins and xenobiotic exposure affects miRNAs as mechanisms of toxicity. Studies for this review were identified using the search terms: “zebrafish microRNAs, zebrafish miRNA, zebrafish miRNA toxicity, zebrafish miRNA toxicology, microRNA toxicology, or miRNA toxicology” on PubMed and Google Scholar. In this review, miRNAs in zebrafish are referred to as simply “miR-XXX” with human miRNAs denoted as hsa-miR-XXX.

2. miRNA regulation of toxin response

Mechanisms of miRNA regulation of toxin response is in its infancy with studies beginning to evaluate mycotoxins and cyanobacteria (Table 1). Wu and colleagues through a series of publications detailed the effects of developmental exposures to the mycotoxins citrinin and ochratoxin, which are contaminants of foods including cereals as well as animal feeds. In evaluating cardiotoxicity, the authors focused on two miRNAs that regulate heart development: miR-138 and miR-218a. Citrinin exposure caused decreased expression of miR-138, which was associated with low survival and abnormal heart development. Although miR-218a expression decreased, no cardiotoxicity parameters appeared to be altered with this miRNA [32]. miR-138 expression in zebrafish regulates gene expression in specific areas of the heart chamber, while miR-218a regulates heart loop formation; so alterations in these genes can lead to heart malformations [33]. Developmental exposure to the mycotoxin ochratoxin resulted in increased expression of miR-731 and was associated with decreased expression of renal genes, renal morphological defects, as well as a decrease in expression of vasculature genes [23,34]. miR-731 is an miRNA that has been shown to be regulated in conditions such as hypoxia, and regulates cell

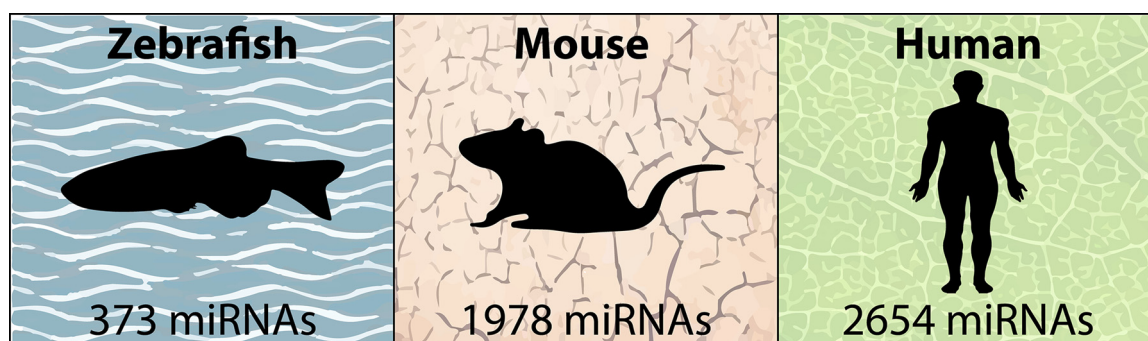


Fig. 2. Total number of mature miRNAs currently identified in zebrafish, mouse, and human genomes. Numbers were attained from the latest version of miRbase release 22.1 (October 2018).

Table 1
miRNA regulation of toxins found to contaminate food and water sources.

Toxin	Time of exposure	miRNAs	Health endpoints	References
50 μ M citrinin	24–72 hpf	miR-138	Decreased expression, leading to lower survival, abnormal heart looping, pericardial edema, cardiac ischemia, miR-138 rescue reduced symptoms and increased survival	[32]
0.5 μ M ochratoxin	6–72 hpf	miR-218a	Decreased expression but didn't effect cardiac endpoints assessed	
0.5 μ M ochratoxin	6–72 hpf	miR-731	Increased expression, leading to decreased expression of renal genes and morphological defects	[36]
		miR-731	Increased expression, leading to decreased expression of vasculature genes and cerebral vasculature loss	[34]
12–48 μ M Microcystin-RR	0.75–24 hpf	miR-146a	Up regulation	
		miR-190b	Down regulation	[37]
		miR-126-DP	Down regulation - Rescue partially restores vascularization	
		miR-430	Up regulation	
		miR-126	Up regulation	

survival by inducing apoptosis [35]. As such, it was proposed that miR-731 upregulation could be blocking DNA replication in renal cells, leading to the declines seen in renal genes in the study.

Cyanobacteria are microorganisms that can produce toxins and can be found in bodies of water, including drinking water sources [38]. Microcystin-RR (MC-RR) is a commonly found toxin in cyanobacterial blooms, and is associated with hepatotoxicity [39]. Early developmental exposure to MC-RR in zebrafish embryos caused a dose dependent increased rate of malformations such as tail defects and decreased vascularization [37]. These health outcomes were linked to changes in expression of 31 miRNAs (Table 1). miR-126 was decreased with MC-RR exposure and rescue was shown to partially restore vascularization. miR-126's role in vascularization is supported in zebrafish and mice [40,41]. Additionally, miR-430 was speculated to play a role in developmental defects, which is supported by rescues of miR-430 in dicer mutants [42]. The results from this study suggest MC-RR toxicity alters several miRNAs, involved in development defects and vascularization [37].

3. miRNA regulation of particulate matter and nanoparticle toxicity response

Particulate matter (PM) is a mixture of solid particles and liquid droplets of trace metals and elements that the US EPA classifies by size [43]. PM composition varies spatially and temporally and their presence in the air is linked to respiratory issues and cardiovascular disease [44]. Zebrafish exposed to PM_{2.5} during development were found to have up regulation of miRNAs involved in DNA repair, hypertension, and immunity [28]. In addition, down regulation of miR-7a/b and miR-19b-3p was observed with the developmental PM_{2.5} exposure [28]. These miRNAs play a role in cardiomyocyte protection and are important for inhibiting cardiac fibrogenesis [45]. An additional study assessed methyl mercury, a component of PM, influences on miRNA profiles at 48 hpf [29]. Decreases in miR-7147 and miR-26a were observed. Increases in miR-375 and miR-206, which regulate pathways for cardiac muscle contraction, were also found [29]. Past studies focusing on PM pollution found differential expression of miRNAs involved in cardiotoxicity with different exposures in other animal models, further validating risk of adverse cardiac health outcomes and align with the findings reported in this zebrafish study (e.g., [45]). These results also suggest that PM exposure results not only in cardiotoxicity, but has potential for increased risks for cancer, inflammation, and immunity (Table 2).

In addition to general PM, miRNA mechanisms of nanoparticle toxicity are also beginning to be identified using the zebrafish model system. Nanoparticles are a microscopic unit of a substance that has a minimum of one dimension less than 100 nm. Nanomaterials are used in medicine, food storage/packaging, and in cosmetics such as toothpastes and sunscreens. Although some nanoparticles are intended for consumption, the health risks for nanomaterials not meant for consumption have become an issue of concern [46]. Currently only one

study, which performed an *in silico* analysis on nanoparticle toxicity, used the zebrafish model [20]. This study evaluated networks related to oxidative stress, DNA damage, and inflammation for nanoparticles of varying chemicals, sizes, shapes, and concentrations. Based on pre-selected criteria, the analysis found six tissue or biological response-specific miRNA-mRNA networks using bioinformatic algorithms for associating miRNA with mRNA (Table 2). Overall, miR-223 was predicted in all three networks and selected as the key regulator for zebrafish tissue damage induced by nanoparticles. miR-223 was indicated as being highly conserved, playing roles in immune cell development including myeloid cells and inflammation regulation. This study identified that the main mechanism of regulation is between miR-223 and insulin like growth factor-1 receptor (*igf1r*) [20,47]. This finding is important because this receptor is involved in cell growth and tumor progression [116].

4. miRNA regulation of environmental chemical toxicity response

4.1. miRNA regulation of pesticide toxicity response

Pesticides are chemicals used to control pests, which can range from animals, plants or fungi. Several studies used the zebrafish to investigate the role of miRNAs in insecticide toxicity. Fipronil is an insecticide that blocks ion channels and triazophos is also an insecticide that works through acetylcholinesterase inhibition. Both of these chemicals have been found as co-contaminants in the same environmental areas. Assessing adult zebrafish exposure for four days, mixtures of these chemicals found miR-29b and miR-738 to be down regulated after adjuvant exposure (Wang et al. 2010). Follow-up studies focusing on fipronil exposure found dose dependent down regulation of miR-155, miR-216b, and miR-499, increasing expression of the target gene cytochrome b561 domain containing 2 (*cyb561d2*), which is important for electron transfer and cell defense [48,49]. Triazophos exposure resulted in dose dependent down regulation of miR-217, which the authors suggest as a potential biomarker for triazophos exposure [50] (Table 3).

Atrazine is an herbicide commonly used in crop fields, and due to concerns over water contamination, the US EPA has set the potable water limit at 3 μ g/l (ppb). An embryonic atrazine exposure in zebrafish resulted in a variety of miRNAs with altered expression [52]. hsa-miR-126–3p, which is implicated in cancer and inflammation [53], was found to be altered at all exposure concentrations. miR-10, which regulates hox genes and can lead to morphological malformations [54], was also reported to have altered expression. Two miRNAs that regulate angiogenesis, miR-23a and miR-24, were also changed. Furthermore, miR-124 expression was decreased. This miRNA plays an important role in neurogenesis [55]. The authors also observed several miRNAs differentially regulated that are associated with various types of cancers [52]. The results from these studies show that a single exposure to pesticides can alter several miRNAs that affect gene regulation and responses to chemical stress (Table 3).

Table 2
miRNA regulation of particulate matter and nanoparticle toxicity response.

Xenobiotic	Time of exposure	miRNAs	Health endpoints	References
100 µg/ml PM _{2.5}	6–120 hpf	let-7b miR-153 miR-122 miR-24 let-7i let-7i miR-7a/b miR-19b-3p	Up regulation - Regulates genes in DNA repair and breast cancer Up regulation - Associated with hypertension in rats Up regulation - Regulates hypoxia based glucose metabolism Up regulation - Roles in immunity and inflammation Down regulation - Role in immunity Down regulation - Cardiomyocyte protective Down regulation - Inhibits cardiac fibrogenesis	[28]
3 mg/mL Silica nanoparticle, 0.01 mg/ml MeHg	48 hpf	miR-7147, miR-26a miR-26amiR-375, miR-206	Down regulation Up regulation (Many miRNAs and genes assessed but the authors focused on these miRNAs that were part of a pathway for cardiac muscle contraction)	[29]
<i>In silico</i> nanoparticles		miR-124 miR-144 miR-19a miR-155a miR-223	Key regulator for Wnt signaling and implicated in DNA double strand damage Key mediator for oxidative stress Important in oncogene regulation and in inflammatory responses Inflammatory response mediator with MAPK and TNF signaling as candidate pathways Predicted in three networks as a key regulator in tissue damage	[20]

4.2. miRNA regulation of other environmental chemicals

A few studies have initiated identification of mechanisms of miRNA regulation in the toxicity of multiple other environmental chemical contaminants including the dioxin 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the antimicrobial agent triclosan, the flame retardant hexabromocyclododecane (HBCD), the perfluoroalkyl substance perfluorooctanesulfonic acid (PFOS), the metal copper, the plasticizers bisphenol S (BPS) and bisphenol A (BPA), and the polychlorinated biphenyl (PCB) 1254 (Aroclor 1254) (Table 4).

TCDD is a contaminant of chlorophenoxy herbicides and a known teratogen, but the mechanism of teratogenicity is not completely understood. To address miRNA dysregulation of TCDD developmental exposure, zebrafish embryos were exposed for 1 h (30–31 hpf) and miRNA profiles determined at 36 and 60 hpf using next generation sequencing (SOLiD) and two different microarray platforms for comparison of the technologies [24]. Surprisingly, the platforms identified dysregulation of different miRNAs, but the two microarray platforms (Agilent and Exiqon) had better overlap in miRNAs and agreement in how those shared targets were differentially expressed. Between all three techniques, only up regulation of miR-27, which is important for craniofacial cartilage development and vascular development, was consistent [63,64] and raises questions on the inconsistency among the platforms.

Triclosan is an antimicrobial agent that the US FDA issued ruling to remove from over the counter anti-microbial soaps [115]. Developmental triclosan exposure in zebrafish causes neurotoxicity such as decreased axonal length, and synaptic density and pathways associated with liver function [65,66]. To understand the role of triclosan exposure on miRNA regulation, zebrafish were exposed during development from 4–96 hpf. Four miRNAs with functions related to fatty acid synthesis and metabolism were up regulated: miR-125b, miR-205, miR-142a, and miR-203a [56]. In another study, triclosan developmental exposure from 6–120 hpf was shown to cause up regulation of miR-137 leading to decreased expression of genes important for neurodevelopment and metabolism [57]. These findings together suggest that triclosan elicits changes in fatty acid metabolism with implications for neurodevelopmental and liver disruption (Table 4).

Hexabromocyclododecane (HBCD) is a flame retardant and is used in polystyrene building insulation [67]. HBCD is an endocrine disrupting chemical and is currently undergoing risk evaluation by the US EPA (2019). Exposures to low concentrations of HBCD during development is associated with increased heart rates and expression alterations in genes that regulate heart rate [68]. To further determine miRNA regulation of HBCD cardiotoxicity, zebrafish were exposed to HBCD during embryogenesis and miRNA expression assessed [23,36].

Altered miRNAs identified in this study were related to cardiac hypertrophy and in regulating cardiac diseases. The authors also associated thicker ventricular walls, collagen deposition, and up regulation of genes associated with the affected miRNA pathways, which agreed with the cardiotoxicity observed [23].

Perfluorooctane sulfonate (PFOS), a perfluoroalkyl substance that was phased out by its major producer in 2002, is still present in environmental sources today due to its persistence. To determine miRNA dysregulation by PFOS exposure, zebrafish embryos were exposed from 6–24 hpf or 6–120 hpf and a microarray platform used to identify altered miRNAs [58]. 39 and 81 different miRNAs were identified at 24 or 120 hpf, respectively. miRNAs affected by PFOS exposure were involved in cell signaling/proliferation, adipose metabolism, and hormone secretion pathways. Targeted gene prediction on the most differentially regulated genes was completed and found that miR-19-b-c targets genes involved in neurodevelopment [58]. The results agree with previous reports that miR-19b acts in a complex to promote neural survival [69] and with the developmental neurotoxicity that is observed in mice with PFOS exposure [70].

The heavy metal copper is a common environmental contaminant that causes developmental toxicity, behavioral hyperactivity in larvae, and a reduction in memory in adult zebrafish [71,72]. Copper is also known as an olfactory toxicant. Focusing on the olfactory system, adult zebrafish were exposed for 24 h to copper and the genome interrogated using the Affymetrix GeneChip miRNA 2.0 array [59]. Results of this study revealed that miRNAs were differentially expressed in a dose dependent manner (Table 4). Based on their findings, the authors predicted that miR-187 has a role in neurogenesis. miR-187 expression has been altered in neural progenitor cells of rats inflicted with stroke, suggesting miR-187 to be important for neural maintenance and disease states [59,73]. miR-140 was changed in all concentrations and plays a role in craniofacial bone and cartilage development [74]. Moreover, 9 of the 11 let-7 isoforms in zebrafish were up regulated, which are important for neurogenesis in the central nervous system. In addition, two miRNAs, miR-128 and miR-138, which are expressed in the olfactory bulb, were also differentially expressed. Overall, several miRNAs with roles in neurogenesis were dysregulated by the copper exposure [59] (Table 4).

Plastic contamination is a growing environmental concern due to the volume of plastic debris present in the ocean, waterways, and animals in the environment [75]. Several papers discuss different types of plastics exposure and altered miRNAs. Lee and colleagues assessed exposure to the plasticizer, bisphenol S, in young adult male zebrafish using the Affymetrix GeneChip miRNA 4.0 Array [60]. 14 miRNAs were differentially expressed by both concentrations utilized in the experiment, and six of these miRNAs target *cyp19ab* mRNAs [60] (Table 4).

Table 3
miRNA regulation of pesticide toxicity response.

Xenobiotic	Time of exposure	miRNAs	Health endpoints	References
0, 4, 4.40, 4.84, 5.32, 5.86 or 6.44 mg/l of 30 % triazophos microemulsifier; 0.050, 0.080, 0.128, 0.205, 0.328 or 0.524 mg/l for 1% fipronil ME; 1.5, 1.8, 2.16, 2.59, 3.11, or 3.73 mg/l for 31 % triazophos + fipronil ME	96 h adult exposure	miR-135c, miR-30b, miR-365 miR-21, miR-31, miR-203b, miR-455 miR-199, miR-22b, miR-499 miR-128, miR-9 miR-735 miR-181a, let-7i, miR-203b miR-155, miR-99 miR-155	Decrease expression with triazophos exposure Increased expression with triazophos exposure Decreased expression with fipronil exposure Decreased expression with mixture Increased expression with mixture Increased expression with ME formulation Decreased expression with ME formulation Dose dependent down regulation - Associated with increase in <i>cyb561d2</i> which regulates electron transfer and cell defense Down regulated and interacts with the gene <i>cyb561d2</i> which was up regulated in a dose dependent manner and is involved in chemical stress Dose dependent down regulation - Suggested as a biomarker for triazophos Dose dependent up regulation at 36 hpf, suppression at 48 hpf, and up regulation at 60 hpf in 30 µg/l treatment group - Associated with hemorrhage, vascular remodeling and maturation effects in zebrafish embryogenesis Altered in all 3 concentrations - Important for tumor suppression in several cancers, angiogenesis, and inflammation miR-10 family regulates <i>hox</i> genes, specifically <i>hoxb1a</i> and <i>hoxb3a</i> which could lead to morphological malformations Regulates angiogenesis Key epigenetic factors in identifying prostate, breast and gastric cancer Highly expressed in several types of cancer Important for tumor suppression	[51]
0.034, 0.068, 0.137, 0.274, 0.548, 1.098 µM fipronil	96 h adult exposure	miR-216b, miR-499		[48]
0.034, 0.068, 0.137, 0.274, 0.548, 1.098 µM fipronil	96 h adult exposure	miR-217 miR-126		[49]
0.45, 0.9, 1.8, or 3.6 µg/ml triazophos 0, 0.3, 3, or 30 µg/l atrazine	1 hpf to 12, 24, 36, 48, 60 or 72 hpf	hsa-miR-126-3p miR-10 miR-23a, miR-24 miR-16a, miR-16b miR-18a, miR-18b-5p miR-143, miR-216b, miR-217 miR-26a miR-124		[50] [18]
			Altered at 30 µg/l - Regulates neurogenesis Decreased expression at 30 µg/l - Important for neurogenesis	

Table 4
miRNA regulation of environmental contaminants.

Xenobiotic	Time of exposure	miRNAs	Health endpoints	References
5 nM TCDD	30 hpf for 1 h Assessed at 36 and 60 hpf	miR-24 miR-27 miR-144, miR-451 miR-23a, miR-204 miR-125b miR-205 miR-142a miR-203a miR-137	Up regulation - Proposed regulation of cardiac myocyte apoptosis and cardiac fibrosis Down regulation - Proposed dysregulation of erythropoiesis regulation and maturation Up regulation - Proposed cardiac hypertrophy Down regulation - Reduced expression of claudin proteins involved in tight junctions Dose dependent dysregulation - Involved in fatty acid synthesis and metabolism Dose dependent up regulation - Involved with DNA repair/cell cycle Dose dependent up regulation - Associated with the P53 signaling pathway Dose dependent up regulation - Associated with fatty acid synthesis and metabolism Up regulation in visceral mass, forebrain and olfactory bulb; Down regulation of regulatory genes <i>bhlHa</i> , <i>mapk6</i> and <i>runx1</i> ; Increasing expression of miR-137 led to bent spines, neuromast decrease, and hypoactivity Down regulation associated with cardiac hypertrophy and downregulation of <i>nkx2.5</i> , which is important for cardiac development	[24]
62.5, 125, or 250 µg/l triclosan	4–96 hpf	miR-219, miR-145, miR-194b, miR-221, miR-146b miR-194d, miR-739 miR-181a miR-19b-c miR-187, miR-140 miR-203a, miR-199*, miR-16a, miR-16c, miR-25 miR-193b, miR-214, miR-183, miR-140*	All up regulated, except miR-146b, which was down regulated and is important for cardiovascular related diseases Up regulated - Associated with inhibiting tumor cell proliferation by repressing p27 Up regulated - Targets genes important in brain and nervous system development Altered in all concentrations Up regulated - Associated with miRNA levels of host genes in Ca signaling Down regulated	[56]
62.5, 125, or 250 µg/l triclosan	6–120 hpf, 6 hpf-90 dpf (for pathology)	miR-137	Down regulation associated with cardiac hypertrophy and downregulation of <i>nkx2.5</i> , which is important for cardiac development	[57]
2, 20, or 200 nM Hexa-bromocyclododecane (HBCDD)	1–72 hpf	miR-1	Down regulation associated with cardiac hypertrophy and downregulation of <i>nkx2.5</i> , which is important for cardiac development	[23]
1 µg/mL Perfluorooctane sulfonate (PFOS)	6 hpf to 24 or 120 hpf	miR-219, miR-145, miR-194b, miR-221, miR-146b miR-194d, miR-739 miR-181a miR-19b-c miR-187, miR-140 miR-203a, miR-199*, miR-16a, miR-16c, miR-25 miR-193b, miR-214, miR-183, miR-140*	All up regulated, except miR-146b, which was down regulated and is important for cardiovascular related diseases Up regulated - Associated with inhibiting tumor cell proliferation by repressing p27 Up regulated - Targets genes important in brain and nervous system development Altered in all concentrations Up regulated - Associated with miRNA levels of host genes in Ca signaling Down regulated	[58]
6.3, 16, or 40 µg/l CuCl ₂	12 mpf zebrafish exposed for 24 h	miR-187, miR-140 miR-203a, miR-199*, miR-16a, miR-16c, miR-25 miR-193b, miR-214, miR-183, miR-140*	Up regulated - Targets genes important in brain and nervous system development Altered in all concentrations Up regulated - Associated with miRNA levels of host genes in Ca signaling Down regulated	[59]
5, 50 µg/l bisphenol S	3–4 mpf adult male zebrafish exposed for 21 d	let-7 a, e, f, g, h, i miR-7a miR-128 miR-724, miR-187, miR-126, miR-30c, miR-16c, miR-203a, miR-203b miR-184, miR-27e, miR-122, miR-204, miR-430a miR-205 miR-30c, miR-192, miR-430b, miR-454b, miR-499 miR-223, miR-363 miR-193b miR-2189 miR-430b miR-724, miR-725, miR-193a, miR-202, miR-205, miR-133a miR-21	Up regulated - Associated with regulation of development and neurogenesis Up regulated - Associated with down regulation of target cytokine signaling 1 Up regulated - Associated with neural differentiation Up regulated - Associated with neurogenesis Uniquely regulated at 5 µg/L Up regulated - Targets <i>cyp19a1b</i> Down regulated - Targets <i>cyp19a1b</i> and genes associated with hematopoiesis and development of lymphoid organs and immune system Down regulated Up regulated Down regulated - Associated with genes regulating cell cycle Upregulated - Associated with apoptosis and autophagy Targets several genes in oxidative phosphorylation, apoptosis, and cell cycle Dose dependent up regulation. - Associated with the loss of calcium in zebrafish skeleton	[60]
100 nM bisphenol A	3 weeks in male zebrafish	miR-223, miR-363 miR-193b miR-2189 miR-430b miR-724, miR-725, miR-193a, miR-202, miR-205, miR-133a miR-21	Down regulated Up regulated Down regulated - Associated with genes regulating cell cycle Upregulated - Associated with apoptosis and autophagy Targets several genes in oxidative phosphorylation, apoptosis, and cell cycle Dose dependent up regulation. - Associated with the loss of calcium in zebrafish skeleton	[61]
0.125, 0.5, 1.0 mg/l PCB 1254	24–120 hpf	miR-21	Dose dependent up regulation. - Associated with the loss of calcium in zebrafish skeleton	[62]

Exposure to a similar plasticizer, bisphenol A, in adult male zebrafish caused up regulation of 14 miRNAs, down regulation of one miRNA, and differential expression of 6188 mRNAs in liver [61]. The authors proposed that 15 miRNAs could modulate 50 % of the altered mRNAs. The miRNA/mRNA deregulated pathways that were top ranked were pathways for nonalcoholic fatty liver disease, oxidative phosphorylation, and metabolic pathways [61]. Together, the results of these two studies suggest that male zebrafish exposed to bisphenol compounds results in alterations of miRNA pathways related to reproduction, metabolism, and liver toxicity (Table 4).

Polychlorinated biphenyls (PCBs) are chlorinated hydrocarbons that were used in various industrial processes until 1979 when they were banned in the US [76]. A developmental exposure to PCB 180 in rats was reported to cause sex-specific alterations in cranial size and teeth size [77]. This is due in part to miR-21's role in regulating osteoclastogenesis and bone metabolism [78]. In agreement with this data, miR-21 was found to be up regulated in a dose-dependent manner in zebrafish exposed to PCB 1254 and to be associated with calcium loss in the zebrafish skeleton [62] (Table 4).

5. miRNA regulation of ethanol toxicity

Ethanol exposure studies are important due to severe developmental effects that are observed in children born to women that consume high amounts of ethanol while pregnant. Several labs have studied the relationship between ethanol exposure and dysregulation of miRNAs and have focused on different physiological outcomes (Table 5). Using a microarray-based approach, Soares et al. [79] identified altered miRNA profiles following exposure to ethanol from 4–24 hpf. Dose dependent differential miRNA expression was observed suggesting different cell responses during development and cell cycle processes (Table 5). miRNAs up regulated in both concentrations were miR-153a, miR-725, miR-30d, let-7k, miR-100, miR-738, and miR-732 [79]. Two other studies evaluating ethanol exposure during gastrulation observed down regulation of miR-9 and miR-153c, which is proposed to occur via methylation, leading to microcephaly, cranial abnormalities, and behavioral hyperactivity [80,81]. Meanwhile, chronic exposure in adult zebrafish for 8 weeks resulted in musculoskeletal damage through down regulation of miR-140–3p [82]. miR-140–3p has been found to target *hey1* and *notch1*, both of which were up regulated in treated zebrafish in this study [82]. The authors proposed that miRNAs that are altered in the Notch pathway induce ethanol-related myopathy, which was supported by the observed 12 % reduction of muscle fiber in the ethanol exposed zebrafish [82].

6. miRNA regulation of pharmaceuticals

Pharmaceuticals, such as antidepressants have been ubiquitously found in aquatic systems, downstream of wastewater treatment plants, and in drinking water sources [83,84]. Antidepressant and anxiety medications are prescribed to improve mood and behavior related disorders, but concerns have been raised over developmental effects

increasing risk for poor motor development in humans and zebrafish [85,86]. Fluoxetine is a medication synthesized by Eli Lilly and is commonly prescribed for depression and anxiety. Zebrafish exposed throughout development to fluoxetine were found to have altered miRNAs in tissues and consistently increased miRNAs in eggs as adults [31] (Table 6). The authors also note their study is the first paper to link toxicant exposure to miRNA expression changes related to egg quality [31]. This finding suggests that a developmental exposure to fluoxetine can change expression of miRNAs in gametes. In another study, adult exposure to fluoxetine at environmentally relevant concentrations was also found to cause similar hepatic miRNA profiles up regulated in metabolic pathways as animals that are fasting [87].

A developmental toxicity study was performed with another drug, valproic acid, which is used to treat epilepsy, mood related mental health issues, and migraines [12]. Alterations in differentially expressed miRNAs were then linked to the presence of morphological and behavioral alterations. Exposure to valproic acid resulted in 35 differentially expressed miRNAs, of which seven were confirmed with qPCR (Table 6). Some of the up regulated miRNAs are involved in cancer and cell cycling pathways, while the down regulated miRNAs are involved in MAP kinase signaling, insulin, and axon guidance [12]. This exposure also resulted in altered behavior in the larvae including circular swimming patterns, twitching, and a reduced startle response. Moreover, morphological abnormalities such as pericardial effusion, decreased pigmentation, and spinal curvature were also observed in the exposed larvae [12].

Another drug prescribed for depression and anxiety, venlafaxine, was assessed for altering miRNA profiles in adult zebrafish at two different temperatures [88]. This exposure resulted in down regulation of miR-22b-3p and miR-301a, but no effect of temperature was found. Both miR-22b and miR-301a are involved in metabolic pathways including drug metabolism [88]. In addition, miR-22 is a conserved miRNA important for proper gonadal function [94,95], which also agrees with the pathways identified to be altered in this study.

Nam and colleagues evaluated developmental liver toxicity with exposure to the drugs tamoxifen and acetaminophen [89]. Tamoxifen is a drug used to treat estrogen receptor-positive breast cancers [96], while acetaminophen is a drug used to commonly treats aches and pains. miR-122 was chosen as a potential biomarker for liver injury in this study. Tamoxifen was reported to reduce liver transparency in larvae, which was associated with decreased expression of miR-122, while acetaminophen exposure increased expression of miR-122 [89]. A separate study also evaluated miRNA expression profiles following an adult exposure to acetaminophen [97]. A dose dependent increase in miRNA expression in the liver was reported (Table 6). Liver toxicity due to acetaminophen and tamoxifen exposure is supported in this study, as well other studies in zebrafish and mice with differential expression of genes related to lipid metabolism and hepatotoxicity [98–100]. Several studies in other animal models have evaluated hepatotoxicity and altered expression of miR-122 after exposure to tamoxifen, acetaminophen, or valproic acid. miR-122 has been identified as a highly conserved and integral regulatory miRNA for genes involved in hepatic

Table 5
miRNA regulation of ethanol exposure.

Xenobiotic	Time of exposure	miRNAs	Health endpoints	References
1 or 1.5 % ethanol	4–24 hpf	miR-153a, miR-725, miR-30d, miR-let-7k, miR-100, miR-738, miR-732	Up regulated in both exposures	[79]
430 μ M ethanol	3.5–7.5 hpf	miR-9	Down regulated - Decreased methylation and decreased expression of <i>fgfr-1</i> and <i>foxp-2</i> leading to teratological defects	[80]
Up to 300 mM ethanol	4–24 hpf	miR-153c, miR-204 miR-9	Down regulated - Role in altered locomotion	[81]
0.5% ethanol	8 weeks in adult zebrafish	miR-140–3p miR-146	Down regulated - Associated with craniofacial skeletal development Down regulated - Targets <i>hey1</i> and <i>notch1</i> , which are upregulated in treated animals Up regulated - Targets Notch signaling to suppress myogenesis	[82]

Table 6
miRNA regulation of pharmaceuticals.

Pharmaceutical	Time of exposure	miRNAs	Health endpoints	References
54 µg/l Fluoxetine	3 – 144 hpf	miR-181a miR-740 miR-26a, miR-30d, miR-92a, miR-103, miR-30d, miR-92a	Up regulated in ovary and fin tissue of exposed females Down regulated in eggs - Targets multiple stress axis transcripts Up regulated in eggs from 5 mpf females; Down regulated in eggs from 9 mpf females - miRNA changes transcripts in eggs with reduction of transcripts in eggs from treated females at 5 mpf and increased at 9 mpf	[31]
540 ng/l fluoxetine	7 days in adult females	let-7d, miR-140–5p, miR-301a, miR-457b	Up regulated - Similar miRNA expression profile of fluoxetine exposed animals and animals that were fasted	[87]
1 mM valproic acid	4–48 or 96 hpf	miR-16a, miR-18c, miR-22a, miR-457b miR-96, miR-122, miR-124, miR-132, miR-135a, miR-140, miR-182, miR-182*, miR-724 miR-10b, miR-10c, miR-16a, miR-16b, miR-18c, miR-22b, miR-210*, miR-217, miR-451, miR-455, miR-457b miR-10b, miR-10c miR-21, miR-27c, miR-34, miR-122, miR-192, miR-194a, miR-724 miR-29a, miR-29b miR-132, miR-212 miR-22b-3p, miR-301a	Up regulated at 48 hpf Down regulated at 48 hpf Up regulated at 96 hpf	[12]
1 µg/l venlafaxine and temperature (27° or 32 °C)	Adults exposed for 21 days	miR-122	Up regulated - Regulates hox genes for anterior posterior development Down regulated at 96 hpf	[88]
0.5, 1, 2 µM tamoxifen	4 dpf for 24 h 3 mpf for 24 h	miR-122	Down regulated - Important for neuron survival Down regulated - Important for neurogenesis	[89]
1, 2, 4 µM acetaminophen	Adult fish (5–24 mpf) exposed for 3 h 6–24 hpf	miR-19a, miR-19c, miR-19d	Down regulated - Gene enrichment shows metabolic pathways such as drug metabolism is altered; Observation not seen after recovery period Decreased miRNA-122 expression with increasing tamoxifen concentration in adults; Increased miRNA expression with acetaminophen exposure	[97]
20–40 mM acetaminophen	Adult fish (5–24 mpf) exposed for 3 h	miR-122	Dose dependent increase in circulating expression, which was associated with hepatotoxicity	[90]
5 nM retinoic acid	6–24 hpf	miR-124, miR-124–3p miR-22a-3p, miR-143, miR-10c-5p, miR-92–3p, miR-148, miR-152, miR-30c, miR-181a-3p, miR-95–5p, miR-184–3p miR-184, miR-96	Decreased expression - Targets <i>cy26a1</i> , which is associated with posterior curved body axis defects Up regulated in 6.25 mg/l treatment; Down regulated in 12.5 mg/l	[30]
6.25 mg/l total composed of: ofloxacin (2.88 µmol/L), ciprofloxacin (3.14 µmol/L), enrofloxacin (2.89 µmol/L), doxycycline (2.03 µmol/L) chlortetracycline (2.02 µmol/L) oxytetracycline (2.26 µmol/L)	4 hpf - 90 dpf	miR-124, miR-124–3p miR-22a-3p, miR-143, miR-10c-5p, miR-92–3p, miR-148, miR-152, miR-30c, miR-181a-3p, miR-95–5p, miR-184–3p miR-184, miR-96	Dose dependent down regulation in eye and otocyst and decreased expression with ISH -Associated with retinal photoreceptor cysts and larval decreased volume of larval otocyst Down regulated in atrium - Associated with atrium tissue damage Down regulated in the liver - Associated with hepatic parenchyma vacuolar degeneration; Down regulated and reduced expression in the heart - Associated with atrial muscle cysts Dose dependent down regulation - Associated with reduced and swollen hepatocytes	[91]
12.5 mg/l of combinations: ofloxacin (5.76 µmol/L), ciprofloxacin (6.28 µmol/L), enrofloxacin (5.79 µmol/L), doxycycline (4.06 µmol/L), chlortetracycline (4.04 µmol/L), oxytetracycline (4.52 µmol/L)	2–120 hpf	miR-10c miR-184 miR-96	Dose dependent down regulation in otoliths - Associated with delayed otolith development; Rescue improved otic vesicle phenotype Dose dependent down regulation in otolith - Injection improved otic vesicle phenotype; Rescue restores hair cell numbers following exposure	[92]
25 mg/l total, composed of ofloxacin (11.5 µmol/L), ciprofloxacin (12.6 µmol/L), enrofloxacin (11.6 µmol/L), doxycycline (8.1 µmol/L), chlortetracycline (8.1 µmol/L), and oxytetracycline (9.0 µmol/L)	6 hpf - 3 mpf in F0 fish	miR-125b, miR-430c miR-124, miR-499	Highly expressed in the liver, brain, gills, reproductive gills and cloaca of F1 larvae at 7 dpf	[93]
6.25 mg/l total composed of: ofloxacin (2.88 µmol/L), ciprofloxacin (3.14 µmol/L), enrofloxacin (2.89 µmol/L), doxycycline (2.03 µmol/L) chlortetracycline (2.02 µmol/L) oxytetracycline (2.26 µmol/L)	6 hpf- 90 dpf	miR-125b, miR-144	Increased expression in F0 adult and F1 larvae at 5 dpf - Target genes identified were <i>ppardb</i> , <i>bc12a</i> , <i>pparaa</i> and <i>pparda</i>	[93]
12.5 mg/l of combinations: ofloxacin (5.76 µmol/L), ciprofloxacin (6.28 µmol/L), enrofloxacin (5.79 µmol/L), doxycycline (4.06 µmol/L), chlortetracycline (4.04 µmol/L), oxytetracycline (4.52 µmol/L)	6 hpf- 90 dpf	miR-125b, miR-144	Increased expression in F0 adult and F1 larvae at 5 dpf - Target genes identified were <i>ppardb</i> , <i>bc12a</i> , <i>pparaa</i> and <i>pparda</i>	[93]
12.5 or 25.0 mg/l total exposure to ofloxacin, ciprofloxacin, enrofloxacin, doxycycline, chlorotetracycline, and oxytetracycline	6 hpf - 3 mpf in F0 fish	miR-125b, miR-430c miR-124, miR-499	Dose dependent increased expression in the F0 adult ovary Highly expressed in the liver, brain, gills, reproductive gills and cloaca of F1 larvae at 7 dpf	[92]

lipid metabolism, hepatocyte differentiation, and overall liver function, making it an attractive miRNA biomarker for liver damage [101–104] as further demonstrated by these studies in the zebrafish (Table 6).

Retinoic Acid (RA) is important for vitamin A synthesis, neurogenesis, and vertebrate body planning. RA is tightly regulated by *cyp26* enzymes [105–107]. In a developmental exposure in zebrafish larvae, RA resulted in down regulation of miR-19a, miR-19c, and miR-19d, which caused posterior curved body defects in larvae through dysregulation of *cyp29a1* [90]. Increases in *cyp26a1*, were also observed in the tailbud with altered expression localized to the dorsal tail tip [90].

β -diketone antibiotics (DKA) are used in human and veterinarian practice to treat infectious diseases. A mixture toxicity study of six DKA drugs: ofloxacin, ciprofloxacin, enrofloxacin, doxycycline, chlortetracycline, and oxytetracycline was completed with exposures spanning embryonic development until adulthood in zebrafish using miRNA sequencing. The expression of miR-96 and miR-184 was found to be altered and at 120 hpf was validated using qPCR, deep sequencing, and *in situ* hybridization for expression in brain, gills, otoliths, and later line neuromast [30] due to their role in regulating various genes in the nervous system [108]. 20 miRNAs highly present and differentially altered were clustered and found to associate with cell cycle and nervous system pathways. DKA exposure was also associated with cyst formation in the adult retina [30]. A follow up study, focused on changes in miR-96 and miR-184 with a developmental exposure to the same pharmaceuticals found delayed otolith development with both miRNAs over or under expressed. The role of miR-96 in hair cell development was validated with a knockout and a rescue by administering a miR-96 mimic [91]. These findings suggest that DKA exposure causes changes in several miRNAs and that miR-96 and miR-184 are key targets leading to malformations of otolith and hair cell sensory organs. To better understand how chronic developmental exposure effects the health of exposed zebrafish (F0) and the subsequent generation (F1), a generational study evaluating developmental DKA toxicity was performed [92]. In the adult F0 generation, histopathological damage was seen in the ovary tissue, as well as increased expression of miR-125b and miR-430c. miR-125b is proposed as an indicator for ovarian cancers [109] and miR-430c has a known role in germ cell differentiation in zebrafish [110]. These functions align well with the histopathological damage and changes in miRNA expression observed in this study. F1 larvae at 7 dpf had high expression of miR-499 and miR-124 [92]. Expression of miR-124 and miR-499 was increased in several regions of larvae, including the brain. These miRNAs are reported to regulate CNS development and to serve as a biomarker for brain injury [111,112], suggesting DKAs may act as a neurotoxicant. A follow-up study focused on miR-125b and miR-144 with the same DKA exposures found increased expression of these miRNAs resulting in lipid retention in the F0 generation and lipid-metabolism-disorder due to more intense lipid accumulation in the F1 generation [93]. Potential gene targets identified were lipid metabolism genes *ppardb* and *bcl2a*, which had decreased expression in the brain, liver, and swim bladder in a dose dependent manner. miR-125's involvement in lipid metabolism agrees with results in human cells and mice, though there is some debate on miR144's role in lipid metabolism [113]. These results suggest that miR-125b plays a role in disease and pathology progression as well in metabolism and ovarian health. The collective results from these studies support DKAs as a developmental and reproductive toxicant in zebrafish with sex specific health outcomes at the genetic, tissue, and morphological level. Adverse health outcomes of DKA exposure can also impact the developmental stages of the subsequent generation [30,91,92,93,114].

7. Current limitations and future directions

miRNAs are a critical epigenetic mechanism in the modulation of gene expression in developmental and disease pathways. As a result, miRNAs are a target of interest in toxicity studies. As discussed in this

review, zebrafish are a good candidate model organism to better understand toxin and xenobiotic toxicity and are being used to readily identify molecular, physiological, and behavioral alterations. More recently, researchers are beginning to apply the zebrafish to define miRNA regulatory mechanisms of toxicity and are grasping a better understanding of strengths and limitations as publications in this research area continue to grow. Currently, many of the studies using zebrafish have applied -omic based approaches to identify miRNAs altered by xenobiotic exposures, but as noted above some studies applying multiple -omic platforms report inconsistent results bringing to question reproducibility. As such, questions still remain on the accuracy of using miRNAs as toxicity biomarkers, but these questions are being addressed as the literature continues to grow on the mechanisms governing miRNA alterations following xenobiotic exposure. For instance, as noted above some studies are beginning to further apply mechanistic approaches in their studies such as performing rescue validation assays to define miRNA mechanisms of toxicity providing greater confidence.

With the limitations noted above, it is expected that future studies will also help to elucidate heritability of altered miRNAs. So far, fluoxetine exposure has been shown to alter miRNAs in the gametes of exposed females and a developmental exposure to DKAs caused developmental health outcomes seen in the next generation. Moreover, it is expected that studies evaluating chemical mixtures and miRNA dysregulation will expand since most often chemical exposures occur in a mixture state with multiple chemicals. To date, triazophos/fipronil mixtures, DKA mixtures, silica nanoparticles, and MeHg mixtures have been performed with the zebrafish. Overall, as miRNA toxicity studies continue it is expected that more answers will be revealed when considering reproducibility and accuracy in defining how miRNAs regulate toxicity and the zebrafish presents as an excellent complementary vertebrate model for these studies.

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The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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