Published in final edited form as:

Green Chem. 2016 December 21; 18(24): 6410–6430. doi:10.1039/C6GC02061E.

ZEBRAFISH AS AN IN VIVO MODEL FOR SUSTAINABLE CHEMICAL DESIGN

Pamela D. Noyes^{1,**}, Gloria R. Garcia¹, and Robert L. Tanguay^{1,*}

¹Department of Environmental & Molecular Toxicology, Oregon State University, Corvallis, OR 97331

Abstract

Heightened public awareness about the many thousands of chemicals in use and present as persistent contaminants in the environment has increased the demand for safer chemicals and more rigorous toxicity testing. There is a growing recognition that the use of traditional test models and empirical approaches is impractical for screening for toxicity the many thousands of chemicals in the environment and the hundreds of new chemistries introduced each year. These realities coupled with the green chemistry movement have prompted efforts to implement more predictive-based approaches to evaluate chemical toxicity early in product development. While used for many years in environmental toxicology and biomedicine, zebrafish use has accelerated more recently in genetic toxicology, high throughput screening (HTS), and behavioral testing. This review describes major advances in these testing methods that have positioned the zebrafish as a highly applicable model in chemical safety evaluations and sustainable chemistry efforts. Many toxic responses have been shown to be shared among fish and mammals owing to their generally well-conserved development, cellular networks, and organ systems. These shared responses have been observed for chemicals that impair endocrine functioning, development, and reproduction, as well as those that elicit cardiotoxicity and carcinogenicity, among other diseases. HTS technologies with zebrafish enable screening large chemical libraries for bioactivity that provide opportunities for testing early in product development. A compelling attribute of the zebrafish centers on being able to characterize toxicity mechanisms across multiple levels of biological organization from the genome to receptor interactions and cellular processes leading to phenotypic changes such as developmental malformations. Finally, there is a growing recognition of the links between human and wildlife health and the need for approaches that allow for assessment of real world multi-chemical exposures. The zebrafish is poised to be an important model in bridging these two conventionally separate areas of toxicology and characterizing the biological effects of chemical mixtures that could augment its role in sustainable chemistry.

*Corresponding author: Phone: 541-737-6514; robert.tanguay@oregonstate.edu.

**Current affiliation: Office of Science Coordination and Policy (OSCP), Office of Chemical Safety and Pollution Prevention, U.S. Environmental Protection Agency, Washington, DC 20460

Financial Interest Declaration:

The authors declare no competing financial interests.

Disclaimer:

The views expressed in this manuscript are those of the authors and do not necessarily reflect the views or policies of the U.S. EPA.

Keywords

Developmental toxicity; Ecotoxicology; Endocrine disrupting chemical; Genomics; Green chemistry; High-throughput screening

Introduction

Humans and wildlife are exposed to an ever-increasing variety of man-made chemicals and complex chemical mixtures. Some of these chemicals have proven to be highly persistent and do not degrade appreciably in humans or the environment, thus presenting long-term exposure concerns and disease susceptibilities. In some instances, they have shown a propensity for long-range transport by the Earth's climate and weather systems being deposited in higher latitudes and some of the most remote places on the planet ¹. In fact, synthetic chemicals are now found in every habitat of the planet and hundreds are detected in a variety of life forms from microbes to plants extending through food webs up to apex predators and humans ²⁻⁵.

Historically, efforts to control chemical releases to the environment have involved technologies and approaches that reduce or clean up releases after the fact. These "end of pipe" strategies, while relevant, are being replaced with advances in pollution prevention technologies that include chemical reagents, processes, and products that are less hazardous and more sustainable. The publication of Paul Anastas and John Warner's important book, *Green Chemistry: Theory and Practice*, in 1998, has expanded the field of green chemistry significantly in the last 20 years and it is now an established scientific discipline ⁶. This book established 12 principles of green chemistry that remain an important organizing framework that guides industry, academic, and government scientists. The pursuit of green chemistry goes beyond waste reduction and pollution prevention, and targets opportunities for design innovation over the entire life cycle of materials (e.g., chemical) to minimize effects on humans and the environment ⁷. Closely related and integrated with the principles of green chemistry and one of its greatest challenges centers on emphasizing and adopting more sustainable chemistry practices in chemical design ⁸⁻¹⁰. This issue is captured succinctly by Collins (2001) in describing man-made chemical design as one that has tended to implement simple reagent designs but by using a vast array of elements. This is contrasted by natural systems that do the opposite, employing just a limited number of environmentally common elements but with a diversity of biochemical processes to select for the desired product or process. With biomimicry in mind, there have been promising efforts to produce more sustainable plastics using zeolites (microporous aluminosilicate minerals) to catalyze the transformation of microbially-synthesized lactic acid to lactide, which is a key precursor to biodegradable polylactic acid plastics that has historically been a costly step in production ¹¹. Alternative peroxide activating catalysts, tetra-amido macrocyclic ligands (TAMLs), have also been designed as oxidation catalysts with a number of structural variants that have shown promise in many uses, including in water disinfection, pulp bleaching, and the break-down of a growing number of persistent chemicals, including chlorinated phenols, explosive residues, dyes, pesticides, and synthetic estrogens ¹²⁻¹⁵. These are but just a couple of examples of looking to sustainable chemistry in designing

functional yet more benign chemicals. However, while these types of efforts are laudable and hold promise, the widespread integration of green and sustainable chemistry into chemical design remains elusive^{16–19}.

In particular, the scale of synthetic chemical production continues to be enormous with thousands of chemicals used today throughout the world as industrial feedstocks, pesticides, pharmaceuticals, and nanoparticles, among many other industrial and household uses. There are currently about 85,000 chemicals that have been produced or imported for sale in the U.S. since chemical inventory tracking was established in 1979 under the Toxic Substances Control Act (TSCA), the primary U.S. statute that oversees industrial chemicals as amended (Frank R. Lautenberg Chemical Safety for the 21st Century Act); however, this does not reflect industrial chemicals currently on the market because it is a cumulative running total. In its most recent data collection, the U.S. EPA reported that roughly 7,700 chemicals subject to TSCA chemical data reporting requirements were produced or imported into the U.S. at more than 25,000 pounds (reporting threshold) during 2011²⁰. By volume this equated to chemical production or importation volumes of about 9.5 trillion pounds per year or 26 billion pounds per day²¹. This U.S. snapshot becomes even more astonishing as it does not include chemicals that are exempt from TSCA or regulated under other statutory authorities, such as pesticides, drugs, food additives, and tobacco products. For instance, in both 2006 and 2007, approximately 5.2 billion pounds and 1.1 billion pounds of pesticide active ingredient were applied globally and in the U.S., respectively, covering several hundred biologically active agents that are regulated in the U.S. under separate statutory authority²². The story is similar in the E.U. where estimates report roughly 100,000 chemicals available for use. Regulations enacted in 2006 under the Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH) program seek to evaluate the safety of approximately 30,000 of these chemicals²³. Similar REACH-types of legislation also have been enacted in Asian countries, including China and South Korea, but the extent of chemical usage is not well described in most countries, particularly those that are less economically developed.

Thus, current statutory and regulatory requirements have resulted in a limited number of environmental chemicals, such as pesticides and some industrial chemicals, being subjected to more rigorous testing and safety evaluation prior to market introduction. It also continues to be challenging for government entities to balance protecting confidential business information (CBI) with ensuring the public's right-to-know so that there is adequate transparency surrounding the composition and safety of chemical products. For the remainder of the many thousands of chemicals being used today generally less is known about their toxicity potential in humans and wildlife. This is not to say that there has not been important toxicity testing of non-pesticides, but oftentimes detailed focus by the broader research community occurs after or in response to chemicals being detected in humans and the environment. Moreover, most human health and ecological effects data used in hazard evaluations for chemical risk assessment continue to focus on direct measurements of apical outcomes of concern, such as reproduction and survival. Analyses typically rely on empirical testing of a single chemical in vertebrate models, such as rodents, with application of uncertainty factors to extrapolate toxicity findings across species and exposure concentrations. Increasingly, these traditional approaches of single chemical testing with in

vivo animals are recognized as impractical as evidenced by the vast resources and time that would be needed to test the enormous backlog of chemicals and environmental mixtures for which less evaluation has occurred and the many new chemistries coming to market each year. Indeed, it has been several years now since the U.S. National Research Council (NRC) recommended that shifts were required in human health toxicity testing from whole animal test systems to *in vitro* methods and bioinformatics to better evaluate biological perturbations and toxicity pathways (Figure 1) ²⁴. Ecological endpoints have also been targeted. For example, a Society of Environmental Toxicology and Chemistry (SETAC) Pellston workgroup examined how to better incorporate mechanistic data into predictive ecotoxicity testing and risk assessment ²⁵. There is also increasing interest in developing test methods and alternative techniques that consider animal welfare and minimize the use of animals in pharmaceutical and chemical testing ^{26, 27}. Thus, the field of toxicology has been shifting from traditional testing methods with whole organisms and single chemical analyses to more predictive-based approaches that strive to characterize early molecular initiating events and biological modes of action (MOAs) to identify chemical and chemical classes that warrant enhanced scrutiny and testing.

This shift in emphasis toward predictive-based approaches and reductions in animal usage in chemical testing has led to increasing interest in the use of *in vitro* and non-mammalian models, particularly embryonic zebrafish, as biosensors to test for bioactivity potential. To help prioritize chemicals for testing, the U.S. Environmental Protection Agency (EPA), National Institute of Environmental Health Sciences (NIEHS), and Food and Drug Administration (FDA) formed a consortium “Tox21” to apply high-throughput technologies to screen roughly 10,000 chemicals and characterize molecular and biological targets and pathways underlying toxicity (<http://www.epa.gov/ncct/Tox21/>). In addition, the U.S. EPA launched its ToxCast™ program in 2007 to further develop HTS technologies with cell-based approaches and embryonic zebrafish to screen chemical bioactivity ^{28–31}. These types of predictive-based test methods with zebrafish provide an opportunity to design and promote inherently safer chemicals and undertake bioactivity evaluations early in the chemical discovery process. The U.S. EPA also has initiated a multi-year transition as part of its Endocrine Disruptor Screening Program (EDSP) toward adoption of HTS and computational toxicology approaches to screen thousands of pesticides and drinking water contaminants for possible endocrine bioactivity in humans and wildlife ³². Likewise, the OECD has an extensive test guideline program in place to support member country efforts to test chemicals for potential endocrine activity of which zebrafish testing is included ³³. Finally, there have been efforts by experts in green chemistry and environmental health to design and implement frameworks to guide testing early in chemical design (e.g., Tiered Protocol for Endocrine Disruption; TiPED¹⁰), reduce laboratory animal use (Alternatives to Laboratory Animals; ATLA ³⁴); and evaluate evidence for endocrine activity (e.g., Systematic Review and Integrated Assessment; SYRINA ³⁵).

This review describes zebrafish testing strategies that are advancing our ability to design safer chemicals. It discusses the rapidly expanding use of the zebrafish model in genetic toxicology, neurobehavioral testing, and as a core *in vivo* model in the fields of HTS and computational testing, and how these technologies can support sustainable chemistry and be positioned to inform early chemical design. While not without challenge, these technologies

are contributing to a rich array of more efficient tools to characterize chemical bioactivity and toxicity pathways across multiple levels of biological organization (Figures 1 and 2). We consider advances in zebrafish testing strategies that are not only expanding our understanding of chemical effects on human health but also among natural biota in ecotoxicology. As such, there is discussion of efforts to use zebrafish as an integrative model in wildlife toxicity screening and in characterizing chemical effects on endocrine system functioning. One of the clear strengths of the zebrafish model is the utility it confers in being able to evaluate chemical effects across different levels of biological organization. The popularity of the zebrafish as a vertebrate model of human disease and chemical toxicity relates to the balance it confers in providing meaningful biological complexity but practical utility as they can be modified genetically and pharmacologically thereby filling an important niche between invertebrate models, such as fruit flies and worms, and more costly mammalian models. Approximately 70% of protein-coding genes and over 80% of disease-related morbidity genes have been shown to have at least one ortholog in zebrafish, making them a genetically tractable vertebrate model to humans ³⁶.

The zebrafish also has generally well-conserved organ systems, tissues, and cell types that make it informative to studying vertebrate development (Table 1). This high degree of conservation makes zebrafish highly applicable to examining chemical effects on embryogenesis that is translational to humans and other vertebrate taxa ³⁷. Moreover, while *in vitro* test systems and ‘omic’ technologies (e.g., genomics) confer many advantages and hold great promise, it can be challenging to reproduce results in animal models as biological complexity, such as toxicokinetics and organ system plasticity, are not easily predicted. Similarly, persistent issues remain in interpreting changes in expression (e.g., transcript levels) as being adaptive or toxic with current *in vitro* systems. Thus, chemicals that elicit activity in *in vitro* assays may require testing and validation with *in vivo* models. Finally, ongoing pressures to reduce the large numbers of animals used in chemical discovery and safety testing have prompted focus on alternative models and zebrafish offer a biologically relevant choice.

Genetic Toxicology and Safer Chemical Design

The use of embryonic zebrafish screens in chemical testing had its genesis in developmental biology with efforts to clarify genes involved in vertebrate development. Some of this work employed small molecules as chemical probes to alter gene functions and products to induce non-heritable phenotypes that could in turn help reveal early developmental processes ³⁸. The rapid pace of advances in genetic screening provides an opportunity to integrate molecular endpoints into safer chemical design and to foster evaluation of the many thousands of chemicals already in use. Methods that allow for control of gene expression can be used to characterize toxic mechanisms pathways so that chemicals can be designed with structural attributes that have inherently lower bioactivity potential.

Traditionally, geneticists used forward genetic approaches in zebrafish and other models to characterize and dissect genes involved in biological processes, notably embryonic development (i.e., phenotype-based relationships). These approaches have sought to identify DNA elements involved in biological processes through the screening of populations of

organisms exposed to a mutagenizing agent that produces random heritable modifications throughout the genome. Some of the first large-scale forward genetic screens using zebrafish led to the discovery of a substantial number of shared genes and pathways essential to vertebrate development^{39, 40}. In contrast, reverse-genetic approaches (genotype-based relationships) involve examining the phenotypic consequences of perturbing the functioning of gene targets. Recent zebrafish genome sequencing/assembly^{41, 42} and large-scale *in situ* hybridization screens⁴³, among other efforts, have revealed thousands of candidate DNA elements that may represent gene targets potentially relevant to chemically mediated bioactivity pathways and thus relevant to chemical design and safety.

In the zebrafish community, morpholino oligonucleotides (MOs) also are a widely used antisense gene knockdown tool to examine toxicity endpoints and disease targets that can be employed early in R&D to design safer chemicals. MOs are ~25 mer nucleic acid bases that are linked to morpholine rings with a neutrally charged phosphorodiamidate backbone that has a high binding affinity to RNA molecules and is stable *in vivo*^{44, 45}. MO applications in zebrafish can act at the RNA transcript level by inhibiting exon splicing⁴⁶ or by blocking translation⁴⁴. Despite their widespread use, MOs have limitations including variability in the degree of knockdown and their transient duration to about three days in early embryogenesis. There is also the possibility for non-specific cell death and other off-target effects, such as p53 inductions, that may produce spurious phenotypes that are not linked to the targeted gene(s) being knocked down. Perhaps most problematic, MO-knockdown approaches in zebrafish have come under increased scrutiny with reports of poor correlation between MO-induced morphants and knockout (KO)-mutant phenotypes^{47–51}.

Several genome editing tools have increased the precision of generating targeted mutations in zebrafish that hold promise in clarifying the toxicity pathways and structure-activity relationships of environmental chemicals. The human engineered zinc finger nucleases (ZFNs)⁵² and transcription-activator-like effector nuclease (TALENs)⁵³ were the first methods developed that allowed for the generation of precise heritable mutations. Both methods create site-specific double strand breaks (DSBs) at targeted locations of the genome. These DSBs are then repaired by sequence homology dependent on independent mechanisms that produce targeted mutational edits. While ZFNs and TALENs have been applied in the zebrafish model, their widespread adoption has been constrained by limited multiplexing capabilities and the considerable amount of time and cost required designing the nucleases^{54, 55}.

The clustered, regularly interspaced, short palindromic repeats (CRISPR)-Cas9 system is a newer genome editing tool with potentially broad applications, including in characterizing the genetic pathways involved in toxic responses that would be highly applicable to safer chemical design. The CRISPR-Cas9 system relies on a single guide RNA (sgRNA) and the Cas9 nuclease to generate targeted DSBs next to specific recognition sites called protospacer adjacent motifs that are followed by DNA repair to produce genome edits. The CRISPR-Cas9 system in zebrafish has been used successfully to generate gene KOs^{56, 57}, disrupt tissue-specific genes⁵⁸, implement single nucleotide substitutions⁵⁹, and introduce exogenous DNA at specific target sites^{60, 61}. It has been shown to be substantially more efficient at generating germline mutations in zebrafish than the ZFN and TALEN systems⁶².

The first HTS CRISPR-Cas9 phenotypic screen of the zebrafish genome targeted 162 loci (83 genes)⁶². This screening study reported a 99% success rate in generating somatic mutations with an average germline transmission rate of 28%.

An important attribute that makes the zebrafish such a well suited *in vivo* model for human translational research for chemical discovery and toxicity screening is the capacity to develop transgenic reporter lines that target specific cells/tissue types, molecular signaling pathways, and physiological processes (Figure 3). Currently, these lines are curated by the Zebrafish Model Organism Database and maintained by ZFIN, University of Oregon (Eugene, OR)⁶³. This work is contributing to a rapidly expanding diversity of zebrafish disease models and drug/chemical screens to understand, prevent, and treat some of the most recalcitrant and costly diseases of our time, including those linked to: psychiatric conditions^{64, 65}; cancers^{66–68}; diabetes and obesity^{69–71}; heart disease^{72–74}; neurodegenerative syndromes^{75–78}; autism⁷⁹; immunodeficiencies^{80, 81}; alcohol, tobacco, drug abuse dependency^{82, 83}; and blood disorders⁸⁴, among many others.

The transcriptome defines the functional and physiological status of an organism and provides information on the gene networks that regulate biological processes. One of the ongoing challenges with zebrafish readouts in chemical toxicity screens is that morphological responses are difficult to decipher because multiple chemicals may elicit the same teratogenic phenotype (e.g., scoliosis, yolk sac edema). Microarray technologies have become useful in hypothesis generating in that they provide an opportunity to dissect toxicological pathways at the transcriptional level in developing zebrafish. Recently, our lab used microarray tools to characterize alterations in the transcriptomes of embryonic zebrafish exposed to the antimicrobial agent triclosan⁸⁵. By phenotypically anchoring the transcriptional alterations to triclosan-mediated developmental malformations, it was possible to propose toxicity mechanisms in zebrafish that may include altered liver development and potential hepatotoxicity. Another related multi-chemical example involved work by Yang and coworkers to apply toxicogenomic methods in embryonic zebrafish to assess whether distinct chemicals could induce specific transcriptional profiles⁸⁶. Gene expression profiles were examined using an oligonucleotide microarray containing 16,399 gene-specific probes. Each of the 11 chemicals tested produced hundreds of genes that were differentially regulated. The expression profiles induced by the chemicals tested were highly specific and could be used similar to a barcode to identify the chemical with high probability. As a final example, to better understand how zebrafish respond to hypoxia, Ton and coworkers used microarray technology to measure the expression changes in >4,500 zebrafish genes in embryos exposed to 24 hours of hypoxia during development from 48–72 hpf⁸⁷. The downregulation of energy consumption has been shown to be a critical defense mechanism against hypoxia in animals⁸⁸. Results from the Ton study showed a strong coordinated downregulation of genes involved in high energy processes, such as protein synthesis, ion pumping activity, and cell division that were induced by hypoxia and reversed upon re-exposure to normal oxygen conditions. These types of microarray studies provided proof-of-principle that the developing zebrafish can be used as a toxicogenomic model to systematically assess the effects of chemicals on gene regulatory networks, and with ongoing development can be positioned for use in chemical discovery and the design of safer alternatives.

However, while microarray continues to be popular and transcriptomic methods can be used to characterize the molecular mechanisms of chemical effects, they continue to be hindered by their inability to distinguish direct from indirect effects of a specific treatment. Next-generation sequencing (NGS) technologies, notably RNA-sequencing (RNA-Seq), have emerged more recently in the zebrafish community as an alternative to microarray. RNA-seq offers several advantages compared with microarrays, such as a more complete snapshot of the transcriptome (e.g., microarrays cannot detect unidentified transcripts and genes), detection of alternative and novel gene isoforms, and coverage of a greater dynamic range at lower abundances. Thus, RNA-seq appears better suited in discerning unique phenotypes that could be missed with microarray as it is not based on *a priori* knowledge of the transcriptome. Several reports have shown that RNA-seq outperforms microarrays in the ability to identify significantly differential gene expression by almost 3-fold^{89, 90}. The increased sensitivity of RNA-seq is attributed to the improved accuracy for detecting low abundance transcripts. In the zebrafish model, a number of RNA-seq studies have advanced our understanding of how the transcriptome regulates vertebrate development by identifying paternally- and maternally-derived transcripts⁹¹, the expressed genes during the maternal to zygotic transition⁹², and the complete set of detectable transcripts across seven different developmental periods⁹³. The information provided by these types of studies can be integrated into toxicogenomic reports to provide a vertebrate developmental network in which to identify chemical mechanisms of action. In zebrafish toxicogenomic research, RNA-seq has been used to reveal conserved biological pathways in 2,3,7,8-TCDD-induced molecular responses in the zebrafish liver compared to *in vivo* mammalian models⁹⁴, identify molecular pathways and biomarkers in response to arsenic exposure⁹⁵, and elucidate the transcriptional responses to oxidative stress in *tert*-butylhydroquinone and 2,3,7,8-TCDD exposed fish⁹⁶.

Improvements in high-throughput genome wide platforms have resulted in a fundamental shift away from protein-centric views of molecular biology. While the number of genes encoding proteins stays relatively constant across a wide range of developmental complexity, the number of non-coding RNA (ncRNA) increases with developmental complexity⁹⁷. Mounting evidence suggests that ncRNAs play significant regulatory roles in complex, multicellular organisms^{98, 99}. As a result, a number of researchers have looked into the role of ncRNA targets in zebrafish, such as microRNAs, to elucidate their role in toxicity pathways^{100, 101} or for use as biomarkers of toxicity¹⁰². In addition to ncRNAs, the expression profiles of proteins¹⁰³, metabolites¹⁰⁴, and DNA methylation patterns^{105, 106} are also being investigated in order to develop a more comprehensive understanding of toxicity mechanisms. Continued efforts to integrate omics data into HTS assays will allow for increased efficiency and a deeper understanding of the links between environmental chemical exposures, toxicity mechanisms, and disease, all of which can promote safer chemical design and evaluation efforts.

HTS Platforms Targeting Early Development

Chemicals will tend to interact with, and if toxic, perturb multiple targets with effects that may manifest as acute, transient, chronic, or delayed depending on the dose, target, age, and physiological status of the animal in relation to its environment. Moreover, animals may

metabolize chemicals to bioactive forms that are more toxic than the parent material, or alternatively may detoxify and excrete chemicals or demonstrate tissue plasticity that may ameliorate adverse effects. Whole organism screens offer the advantage of a more integrated characterization of chemical bioactivity (Figure 1) thereby avoiding some of the inevitable mechanistic bias of single compound-target pairings and cell-based approaches.

The current state of drug discovery demonstrates some of the advantages conferred by *in vivo* systems in optimizing chemical design and evaluation. Specifically, phenotypic-driven screens with whole animals have demonstrated a higher success rate in identifying promising drug therapeutics than target-based approaches that use *in vitro* and cell culture systems¹⁰⁷. Although target-based approaches have yielded many thousands of candidate molecules, this has not translated into an increase in drug discovery. About 40% of new candidate molecules fail during preclinical toxicological safety evaluations at great expense and the sacrifice of many test animals¹⁰⁸. The reasons for the high failure rate of target driven approaches are undoubtedly multifaceted and relate to factors such as the inability to model toxicokinetics and off-target effects in an *in vitro* system. Additionally, these approaches have limited capacity to predict whether modifying a specific target will ameliorate a downstream disease phenotype. These challenges in drug discovery are informative to green chemistry and safer chemical design as they demonstrate some of the limitations of cell-based approaches and the ongoing importance of *in vivo* models, particularly those like zebrafish that fill a niche between *in vitro* and higher vertebrate testing.

An ever-increasing number and variety of low, medium, and higher throughput *in vivo* screens with zebrafish embryos have been and continue to be developed that target an increasing variety of pathways and endpoints (e.g., teratogenicity, endocrine disruption, cardiotoxicity, etc.). The implementation of these screening formats with zebrafish has accelerated dramatically in recent years to promote the design of safer chemical alternatives (Figure 2). While it is not possible to discuss all the many zebrafish assays that have been employed in toxicity evaluations, several approaches are notable due to their relevance to safer chemical design and given the range of chemical structures they aim to consider. They are now being used to test numerous environmental toxicants, pharmaceutical agents, and chemical libraries across a range of life stages, transgenic and mutant lines, test concentrations, and exposure durations¹⁰⁹. One of the clear advantages of zebrafish HTS designs is that only very small amounts of test compound in the microgram or microliter range are typically needed, whereas studies in mammalian assays can require upwards of several hundred grams of compound depending on study design and duration. In addition, zebrafish have been shown to be relatively non-responsive to the carrier solvent dimethyl sulfoxide (DMSO)¹¹⁰. This tolerance to DMSO has made it possible to test an array of chemical structures, including many higher MW chemicals with hydrophobic functional groups that would not ordinarily solubilize in aqueous media. These favorable attributes become especially relevant in early screening of chemical libraries and structures as part of early R&D testing where typically only very small amounts of compound are synthesized with any number of different structural moieties conferring different physicochemical and biological properties.

HTS methods that use embryonic zebrafish are generally consistent with one another in that they use a multi-well plate format to test chemical effects on embryonic development and by assessing mortality and deformities across a range of phenotypes, chemical structures, and concentration ranges (Figure 4). Medium and higher throughput toxicity screening assays with zebrafish embryos have been developed in the U.S. as part of the U.S. EPA-National Center for Computational Toxicology (NCCT) ToxCast program^{28–30, 111}. As a part of this effort, for instance, Padilla and coworkers conducted a developmental toxicity study with embryonic zebrafish to screen ~300 chemicals (i.e., mostly pesticides) comprising the Phase 1 ToxCast chemical library³⁰. Larvae were scored for survival and overt malformations at 6 dpf. A subsequent study conducted in our lab by Truong et al. (2014) used a similar format to the Padilla lab with some differences. Truong et al. (2014) evaluated over 1,000 chemicals that included the Phase 1 ToxCast chemicals tested by Padilla et al. (2012) plus the several hundred chemicals in the Phase 2 ToxCast library. Padilla and coworkers used intact chorionated embryos exposed by static renewal for five days with evaluations on day six, while Truong and coworkers used dechorionated embryos exposed by static non-renewal with evaluations on days one and five. Test concentration ranges were similar, but the Truong study used larger sample sizes and targeted more phenotypes. In terms of screening, Padilla et al. (2012b) ranked and scored malformations based on severity to calculate half-maximal activity concentration (AC₅₀) values; Truong et al. (2014) scored deformities as binary, either present or absent, and computed lowest effect levels (LELs).

While the methods implemented and results in the Padilla and Truong studies digressed both observed similar results. Padilla et al. (2012) found that 62% of the ToxCast Phase 1 chemicals were toxic at one or more concentrations at or below the highest concentration tested (80 μ M). Likewise, Truong detected toxicity in 60% of the Phase 1 chemical library measured as a positive hit for mortality and malformation across one or more concentrations at or below the highest concentration tested (64 μ M). The high percentage of toxic outcomes was expected given that most of the ToxCast Phase I chemicals are pesticides. In comparing positive hits across the two studies, 75% of chemicals scored as toxic in the Truong study were also scored as toxic in the Padilla study, suggesting good concordance across the two platforms but with differences likely attributable to study design. For example, retaining or removing the chorion and variable exposure conditions would be expected to influence the bioavailability and internal dosimetry across the two studies depending on the chemical. Nonetheless, it appears that for chemicals with expected bioactivity, the more limited phenotypic screening by Padilla and coworkers was able to identify chemical-induced developmental abnormalities. Questions remain for compounds with unpredicted or unknown bioactivity that may require more rigorous screening. For example, Truong et al. (2014) identified early notochord deformities in embryos exposed to thiocarbamate pesticides that may not have been identified with a more limited phenotypic screen. Thus, there continue to be important considerations as to the breadth and depth of phenotypic screening to balance appropriate rigor (i.e., avoiding false negatives and positives) with maintaining speed and screening capacity.

Similar zebrafish HTS platforms of early development have been used in the design, testing, and evaluation of new chemistries, notably engineered nanomaterials (ENM)^{112–117}. A major challenge with ENM design centers on identifying features that not only confer

desired performance but also minimize toxicity potential. The enormously varied and rapid pace of new ENM structures makes it impractical, absent great time and cost, to conduct extensive in vivo-based safety testing without dramatically slowing R&D. Optimizing the biocompatibility of ENM is not a trivial matter as their elemental composition, surface functionality, core size, and purity, among other features, may vary enormously and are technically difficult to characterize. HTS platforms with embryonic zebrafish have shown utility in ENM design as they have been integrated into other platforms intended to characterize the structural features of ENMs. For example, HTS platforms with embryonic zebrafish have been combined with other design methodologies to characterize the physicochemical features (e.g., charge, core size) of different gold nanoparticles (AuNP) that impair development (Harper et al., 2011). Characterizing structural attributes that confer bioactivity can be used as a framework for incorporating safety measures into ENM design to broadly identify structural features in ENM that are not desirable.

These types of screening methods have clear utility in identifying chemicals with heightened or reduced bioactivity that could serve as a useful approach for prioritizing chemicals for more testing and to facilitate the design of safer alternatives. One key challenge with characterizing toxicity results from large, structurally diverse chemical libraries relates to dissecting potentially related responses and common toxicity mechanisms among a data-rich and complex set of phenotypes across a range of structures. To begin to address these challenges, a recent study published by our lab ¹¹⁸ applied the morphometric screening techniques by Truong et al. (2014) and integrated results of two locomotor behavioral assays of photomotor responses to characterize the toxicity of over 40 structurally diverse flame retardant (FR) chemicals and their metabolites ¹¹⁸. Hierarchical clustering and principal component analysis (PCA) were employed to evaluate interactions and differences in bioactivity across the morphological and behavioral platforms to discern chemical classes and structural features that confer elevated bioactivity (Figure 5). Results of this study measured FR bioactivity in one or more of the assays and across one or more test concentrations, and found that organophosphate FRs with isopropyl, butyl, and cresyl substituents on phenyl rings were especially potent. In sum, this type of integrated HTS approach not only pointed to ongoing concerns for the safety of FRs in use but provided approaches that could be helpful in designing FRs with intrinsically lower bioactivity potential.

Other combinatorial approaches have also been employed to integrate zebrafish HTS with other high content platforms. For example, a large number of organophosphate FRs were recently tested with a battery of HTS platforms that included zebrafish high-throughput methods similar to those conducted in the Padilla, Truong, and Noyes studies, as well as a divergent set of in vitro assays ¹¹⁹. A point of departure (POD) approach was implemented to compare the relative activity of flame retardants tested across the different test platforms. In addition to efforts to rapidly compare effects across multiple platforms, there have been advances in zebrafish technologies that allow for rapid three-dimensional imaging and phenotyping ¹²⁰. One of the ongoing limitations of many zebrafish HTS approaches is that most embryonic screening involves the manual examination of chemical effects against a set of developmental defects. This aspect of HTS can be time-consuming and subject to variability depending on the reviewer and lab. There have been promising efforts recently

with advanced optical imaging platforms, such as optical projection tomography (OPT), to automate *in vivo* phenotyping of developing zebrafish embryos^{120–122}. Ongoing advances in imaging and analysis of different developmental phenotypes should augment the speed and reproducibility of zebrafish HTS platforms.

Ecotoxicology Testing

The use of traditional lab models in ecotoxicology has proven to be time and resource intensive, logistically challenging whether conducted in the field or laboratory, and difficult to translate from the lab to wild populations and across species. As a result, like in human health, there has been an accelerating shift from empirical methods to pathway-based methods that rely more on predictive tools and models. Some of these methods seek to characterize putative adverse outcome pathways (AOPs) to describe the molecular initiating events (MIEs) and cascades of intermediate key events (KEs) that may culminate in an adverse outcome, such as impaired reproduction or population declines^{25, 123, 124}. In this context, the zebrafish is becoming a useful translational vertebrate model to study chemical bioactivity potential for ecological risk assessment. Perhaps one of the more directly applicable efforts relates to the EU REACH initiative to implement the embryonic zebrafish as a test model to replace fish acute toxicity testing requirements^{26, 125}. REACH has been the subject of criticism due to the predicted increase in animal testing it triggers and ongoing concerns surrounding how fish experience pain and duress^{126, 127}. The fish embryo toxicity (FET) screen with zebrafish was developed in part as an alternative model to be responsive to these animal welfare concerns. Additional benefits of FET screens, like with HTS assays more broadly, include that they are efficient and require only small amounts of chemical.

The OECD guidelines for zebrafish FET testing were finalized and adopted in 2013¹²⁸, although FET approaches have been used in Germany since 2005. The guidelines are straightforward and require that newly fertilized zebrafish embryos be exposed to test chemical for 96 hours with microscopic examinations every 24 hours for lethality and other indicators of failed developmental progression, including embryonic coagulation, impaired somite formation, non-detached tail buds from yolk sacs, and lack of a heartbeat. The performance of the zebrafish FET assay in reproducing acute fish toxicity testing results (mined from U.S. EPA ECOTOX and ECETOC Aquatic Toxicity (EAT) databases) was quantified recently for about 140 pesticides, feedstocks, and other chemicals representing a variety of chemical structures¹²⁹. The results measured as half-maximal effect concentration (EC50) values were generally highly correlative across the testing platforms, supporting its adoption for fish ecotoxicity testing. Efforts have been made to try to extend the acute FET assay to include gene microarray analyses for assessing chronic fish toxicity endpoints but overall these tools remain limited to acute endpoints^{130, 131}. Thus, like with the zebrafish HTS platform more broadly, the FET assay appears to be poised as a useful approach for ecotoxicity applications and chemical design.

The characteristics that make the zebrafish an excellent model for predictive human health assessments are also directly relevant in the context of ecological risk assessment. While historically human health and ecological effects have been assessed using distinct testing methodologies, both disciplines are moving toward predictive approaches that take

Author Manuscript

Author Manuscript

Author Manuscript

advantage of our increasing knowledge of biological pathway conservation. Perkins and coworkers recently published approaches that use pathway-based POD data and benchmark dose modeling from embryonic zebrafish exposed to the developmentally toxic pesticide flusilazole to derive human dosing values ¹³². These values in zebrafish aligned with those derived from more conventional rodent models and provide some demonstration of how zebrafish can be used to assess chemical risk. The zebrafish may prove to be especially valuable for examining chemical effects on aquatic wildlife because it is increasingly feasible with HTS screens and genetic testing to target multi-chemical exposures and non-chemical interactions ^{133–135}. For instance, developmental defects leading to cardiac toxicity and heart failure are a well-described sensitive target for the effects of some petroleum-derived PAHs and their mixtures ^{135, 136}. Hicken and coworkers used zebrafish to show how low levels of PAH exposures to embryos interfered with genes involved in heart development that in turn led to reduced swimming performance and changes in cardiac ventricular morphology in adult fish ¹³³. This mechanistic work with zebrafish is important because it clarified a potential pathway leading from delayed individual toxicity to potentially impaired population fitness among wild fish populations exposed to PAHs by oil spills, hazardous waste sites, and other exposure pathways.

Chemical Screens for Endocrine Activity

Author Manuscript

Endocrine disruption caused by chemical exposures has been the subject of intensive research and continues to be a concern among many environmental and public health scientists and government agencies ^{32, 137–143}. To date, toxicity studies of potentially endocrine active substances have emphasized the brain-gonadal axis and the brain-thyroid axis. Relatively fewer studies have examined the potential for chemically mediated endocrine activity beyond the gonadal and thyroid axes, and even less have focused on the cross-talk underlying hormone regulation and signaling and how chemicals might interfere with these permissive feedbacks. Some evidence may also support perturbations of the vertebrate endocrine systems at low levels of chemical exposures along with non-monotonic dose-response relationships ^{141, 144, 145}. Hormonal systems are involved in many biological responses that are life-stage specific; thus homeostatic perturbations may have profound or transient consequences depending on the age of the organism. The critical importance of thyroid hormone in early fetal development contrasted by the reversible effects (e.g., weight gain) of thyroid hormone insufficiency in adults demonstrates this relationship ¹⁴³.

Author Manuscript

With the rapid advances in genetic testing that allow for characterization of chemical MOAs, the zebrafish has gained prominence in endocrine toxicology as the vertebrate endocrine system, inclusive of the hypothalamus, pituitary, thyroid, pancreas, adrenal gland (fish interrenal organ), ovaries, and testes, are evolutionarily conserved and generally comparable ^{146–149}. Although important differences exist between reproduction in mammalian and non-mammalian vertebrates, reproduction in jawed vertebrates is controlled by the hypothalamic-pituitary-gonadal (HPG) axis and the structure of this endocrine system is shared. Sex steroid hormones are produced primarily in the gonads of both fish and tetrapods with a synthesis pathway that involves gonadotropin-activated signal transductions, cholesterol mobilization and transport, and a multistep enzymatic conversion of cholesterol to steroid hormone. Rate limiting steps in steroid production are mediated by production of

the steroid acute regulatory (StAR) protein involved in cholesterol transport as well as aromatase, which is a member of the CYP family and regulates estrogen biosynthesis. Both StAR protein and aromatase have been shown to be phylogenetically conserved^{150–152}. The general architecture and functioning of the thyroid system is also shared among vertebrates and includes the tightly controlled synthesis of thyroid hormone by the hypothalamic-pituitary-thyroid (HPT) axis and its homeostatic regulation in circulation and target tissues by the activity of iodothyronine deiodinase (Dio) enzymes and other processes. Thus, numerous research efforts have capitalized on this shared biology and used zebrafish to elucidate mechanisms by which chemicals may alter normal endocrine functioning^{153–159}.

While it is beyond the scope of this review to describe all aspects of zebrafish use in characterizing chemical-induced endocrine activity, several approaches are highlighted specifically because they demonstrate how this model can facilitate the design and evaluation of inherently safer and more sustainable chemicals. For example, methods involving transcriptomic analyses of embryonic zebrafish have been employed to identify putative estrogen and androgen responsive genes with exposures to hormones and endocrine active synthetic chemicals^{160, 161}. There are also an increasing variety of zebrafish transgenic fluorescent reporter lines that have been developed to assist in visualizing and characterizing the effects of potentially endocrine active chemicals on brain-gonadal signaling pathways, including *cyp19a*^{162–164}, vitellogenin egg precursor protein¹⁶⁵; growth hormones¹⁶⁶; estrogen receptors^{167–169}; gonadotropin releasing hormone (GnRH) signaling¹⁷⁰; and glycoproteins encoding follicle stimulating hormone (FSH) and luteinizing hormone (LH)¹⁷¹. The development of zebrafish transgenic models now extend to other components of the endocrine system that are possible targets of chemicals, including those linked to the brain-thyroid axis^{171–176}, endocrine pancreas development and functioning^{177–179}, and adrenal-stress responses^{180, 181}.

Differences in fish and mammal endocrine signaling pathways also can be positioned to further advance our understanding of hormonal and chemical effects on other important biological processes. Questions have been raised about the linkages between neurogenic pathways and aromatase activities in neurological disease, including the role that xenoestrogen exposures may play. Estrogens, in addition to controlling reproduction, have been shown to have extensive and measurable effects on neurogeneration and neuroplasticity in many parts of the brain¹⁸². Zebrafish have shown a remarkable ability to repair and renew their brains (and other tissues) after traumatic damage in contrast to mammals that have very limited regenerative competencies^{183–185}. Moreover, the neural tissues of adult teleost fish have been shown to have 100–1000 fold higher estrogen-synthesizing aromatase activity than in corresponding neural tissues of mammals, including humans¹⁸⁶. Two distinct genes encoding aromatase enzyme, namely *cyp19a* and *cyp19b*, have been isolated in zebrafish with *cyp19b* being expressed mostly in the brains and *cyp19a* expressed in the gonads^{187–189}. Expression of *cyp19b* and aromatase B protein in the brain has been restricted to radial glial cells of adult teleosts¹⁹⁰. Radial glia are increasingly recognized as progenitor cells that not only are the source of brain neurons during development but are key to ongoing neurogenesis in adult animals¹⁹¹. Thus, taken together, the zebrafish may prove to be an important model in exploring the relevance of aromatase and estrogens in tissue

repair and regeneration signaling programs, including how endocrine active chemicals and drug agents may hinder or enhance these effects^{191–194}.

More broadly, there have been efforts to design protocols that integrate zebrafish testing with other approaches to evaluate the potential for chemicals to interact with the endocrine system. The Tiered Protocol for Endocrine Disruption (TiPED) protocol is one such effort. It describes a step-wise approach, ranging from in silico tools to in vitro assays and whole organism studies, including with zebrafish, to inform chemical design efforts that minimize endocrine activity¹⁰. TiPED is intended to foster more sustainable chemical discovery under a non-regulatory framework by providing a tiered methodology for interrogating chemicals for potential endocrine activity. For example, this framework was implemented to evaluate several TAML activators, which are proposed alternatives for water treatment and as oxidizers in breaking down synthetic estrogens and some persistent organic chemicals. As a part of this effort, embryonic zebrafish HTS platforms were implemented to screen these compounds for developmental toxicity. TiPED is an innovative approach for integrating chemistry and toxicology that could serve as a model for guiding chemical design to mitigate against other potentially chemical mediated adverse outcomes (e.g., diabetes, carcinogenicity). It could also be expanded to other endpoints and bioassays that may be indicative of potential chemical bioactivity. For example, zebrafish behavioral assays have also been applied in a limited manner to examine the effects of endocrine active chemicals on possible stress and anxiety endpoints using a ‘novel tank’ test¹⁹⁵. The novel tank test has been developed to measure behavioral responses to anxiety (e.g., diving, delayed habituation, thigmotaxis) in zebrafish exposed to chemicals^{195–197}. These behavioral assays have been used by Cachat and coworkers to quantify behavioral indices of anxiety in zebrafish (induced by stress or chemical) and to integrate measurements of whole body cortisol¹⁹⁵. Reider and coworkers employed this type of novel tank test and reported that chemically-induced hypothyroidism with the goitrogen methimazole exacerbated anxiety (latency in exploration/habituation) in zebrafish larvae¹⁹⁸. In another example, zebrafish behavioral testing has been useful in describing how the potential estrogenic properties of BPA could manifest in a complex array of altered behaviors. BPA is a suspected endocrine active chemical that has been shown in some testing to behave like an estrogen mimic^{199, 200}. Recent evidence in BPA-exposed zebrafish identified sex-specific differences in behavioral responses in that male fish were less active (swimming distance, territoriality, and aggression) and circadian rhythms were perturbed in comparison to control males²⁰¹. No significant BPA-linked behavioral effects were reported in females in this study. These types of studies demonstrate the potential for zebrafish behavioral assays to provide a fuller understanding of chemical effects on endocrine-linked anxiety responses and other behavioral domains. However, the results continue to be difficult to interpret. For example, the extent to which anxiety and other behavioral responses in zebrafish are analogous to those of higher vertebrates remains to be clarified. Further studies are also needed to better understand how different zebrafish strains and mutant lines respond in behavioral testing. Another obstacle involves controlling for subtle morphological changes (e.g., musculoskeletal deformities) that might not be detected upon visual inspection but nonetheless could influence fish motor responses. Nonetheless, with continued work,

behavioral testing in the zebrafish holds promise in providing a fuller picture of chemical MOAs that proceed in part or substantially through the endocrine system.

Behavioral Testing in Zebrafish

A number of efforts are underway to develop higher throughput behavioral test methods that use embryonic and larval zebrafish to characterize chemical effects on early neurobehavioral responses that could be highly informative to safer chemical design efforts. One promising area of chemical-behavioral testing involves using embryonic zebrafish (~6 to 120 hpf) as part of developmental neurotoxicity screen to examine chemical effects on early sensory-motor system patterning of the developing nervous system. Starting at 17–19 hpf, zebrafish begin to spontaneously contract their tails reflexively with advancing development of the sensory-motor system²⁰². This response has been shown to be highly sensitive and excitatory to light through non-ocular photoreceptors and neuronal pathways activated in the caudal hindbrain and that may involve opsin-based signaling²⁰³. Targeting this non-ocular response, photomotor response (PMR) platforms with embryonic zebrafish have been designed and validated with large chemical libraries, including approximately 14,000 neuroactive drugs²⁰². Briefly, they are rapid assays that involve using a multi-well plate format with chemically-exposed embryos, typically at 24 hpf, and measuring tail contractions and flexions upon short pulses of intense light followed by darkness. Using this type of HTS format and behavioral phenotyping, Kokel and coworkers found that different structural and functional classes of neuroactive chemicals clustered and elicited specific and reproducible behavioral phenotypes in embryonic zebrafish²⁰². For example, chemical psychostimulants and anxiolytics increased and decreased motor activity, respectively, throughout the test and regardless of whether light or dark was applied. Dopamine agonists lengthened PMR latency periods, and serotonin reuptake inhibitors showed brief but robust responses to light and even caused stimulated activity to a second light stimulus.

Our laboratory in collaboration has also instituted similar PMR assay designs to test environmental chemicals, including screening the PMR responses of embryonic zebrafish exposed to the roughly 1,000 chemicals in the U.S. EPA's Phases 1 and 2 ToxCast libraries²⁰⁴. Chemicals that caused light-dependent and -independent effects on embryonic movement in this assay predicted teratogenicity later in older larvae at 5 dpf. In further demonstration of its utility in safer chemical design, this embryonic PMR assay was one of two behavioral assays used recently to test a suite of FR chemicals with variable structural attributes, and was integrated into a platform that also measured PMRs and morphometric responses in larvae at 5 dpf¹¹⁸. Consistent with observations by Reif and coworkers, the presence or lack of PMR effects in 24-hpf embryos exposed to FRs was predictive of survival and teratogenicity detected later in larvae at 5 dpf. Specifically, the 24-hpf PMR assay predicted the presence or absence of morphological defects for approximately 80% of the FR chemicals examined at 5 dpf. When combined with PMR testing of larvae at 5 dpf, the concordance increased and the presence or absence of 24-hpf and 5-dpf PMR effects predicted 5-dpf teratogenicity for 93% of the flame retardants tested.

Other behavioral screening methods have been applied to take advantage of these earliest movements of embryonic zebrafish. For instance, chlorpyrifos insecticide and other well-

known developmental neurotoxicants have been used as training sets to guide and validate embryonic zebrafish spontaneous tail contractions for use in developmental neurotoxicity screening ²⁰⁵. Raftery et al. (2014) used a 384-wellplate format and exposed transgenic embryonic zebrafish (*flil:egfp*) from 5–25 hpf to 16 chemicals from the U.S. EPA ToxCast Phase 1 library ²⁰⁶. This study employed enhanced green fluorescent (eGFP) stably expressed in the vascular epithelium of this transgenic line to measure spontaneous tail contractions as an early indicator of developmental neurotoxicity. In this study, tail contractions were absent but no gross morphological defects were observed among embryos exposed to abamectin insecticide from 5–25 hpf. This absence of movement is consistent with other studies showing abamectin neurotoxicity being linked to it agonizing GABA receptors that stimulates release of GABA neurotransmitter and produces paralytic responses ^{207–209}.

A number of larval zebrafish screens of behavior have been developed, such as measuring preferences, aversions, and locomotion during alternating periods of light and dark. Zebrafish larvae display consistent patterns of visual locomotor activity upon alterations between periods of light and dark, and have been shown to be dark aversive ^{210–212}. When light is removed a pronounced increase in locomotion occurs that gradually subsides as darkness continues. While the underlying reason for this behavior is still not well described, it has been postulated to be adaptive responses to avoid predators and forage for food. Specifically, evolutionary survival pressures in minnows such as the zebrafish are thought to have given rise to extensive and rapidly developing sensory-motor behaviors, such as saccadic eye movements, optomotor reflexes, rheotaxis, startle-escape locomotion, olfactory and feeding behaviors, circadian rhythms, learning, and memory ²¹³. Thus, the rapidly increasing locomotion observed when larval zebrafish are subjected to darkness has been suggested to be a tractable measure of anxiety, and the decline in movement that is typically observed as darkness continues is proposed to represent habituation ^{214–217}. PMR assays in zebrafish larvae are now becoming increasingly standardized in application although test regimes may vary depending on the goals of individual studies. Typically, movements of chemically-exposed and control larvae are tracked using a closed box that has a multi-well plate holder, internal lighting system for applying stimuli, and a mounted video camera and software to track and integrate movements for subsequent analysis. These types of larval zebrafish assays have been used in HTS platforms to examine neurobehavioral responses and in some cases underlying neurotoxicity mechanisms for a variety of chemicals, including ethanol ^{218–220}, nicotine ²²¹; plastic components and additives ^{222, 223}; nanoparticles ^{117, 224, 225}, fluorinated surfactants ^{226, 227}, flame retardants ^{118, 228–230}, pesticides ^{231–233}, and pharmaceutical agents ²¹⁹.

Beyond PMR assays, other locomotor assays and cluster analyses in larval zebrafish have been able to predict targets for chemicals using behavioral profiling. For example, Rihel et al. (2010) developed a HTS platform of rest/wake cycles with larval zebrafish and applied it to over 5,600 psychoactive drugs to identify important clustering patterns representing relationships between behavioral phenotypes, chemical structures, and biological targets. They showed that neuroactive drugs with different neuro-mechanisms of action (e.g., serotonergic, adrenergic, dopaminergic) elicited distinct behavioral phenotypes. Hierarchical clustering revealed that drugs with correlated behaviors shared common targets and

therapeutic mechanisms, allowing in turn for the proposal of targets of chemicals with poorly understood modes of action. For instance, amitraz insecticide, which is used to treat tick and mite infestations in pets and farm animals, clustered with other α_2 -adrenergic agonist drugs, such as clonidine and guanabenz, reinforcing evidence that this pesticide targets α_2 -adrenergic receptors and the sympathetic nervous system. Thus, it is clear how continued progress with these types of behavioral platforms could be highly applicable in designing safer chemicals with minimized bioactivity.

Like with developmental life stages of zebrafish, the adult zebrafish has also become a popular model to probe how behavioral and neurobiological endpoints are impacted by chemical exposures. A range of behavioral tests have been designed to target different domains associated with sensory-motor systems, cognitive functioning, and even those more subtle responses related to learning, memory, and anxiety. Indeed, zebrafish adults and juveniles have been shown to display a variety of complex behaviors, such as shoaling and schooling^{234, 235}, kin recognition^{236, 237}, territoriality²³⁸, associative learning^{239–241}, and non-associative responses (e.g., habituation)²⁴²; however, as with the neurosciences broadly, our understanding of vertebrate and zebrafish neuroethology and how chemical exposures in turn may cause brain pathologies that produce maladaptive behaviors is an area with many unknowns.

Challenges Going Forward

While advances in zebrafish testing provide opportunities to predict and characterize chemical structure-activity relationships that promote chemical design for reduced bioactivity, it is important to continually evaluate and choose the most appropriate model for toxicity testing based on research goals and the advantages/limitations of the test organism or assay. For instance, maximizing the use of zebrafish in neurotoxicity testing will require continuing to expand our understanding of the relationships between the structure and function of the CNS and PNS of zebrafish and higher vertebrates. This does not negate the use of zebrafish in characterizing chemical effects on the developing or mature nervous system, but rather points to an area where understanding the homologies and distinguishing aspects of brain and neurological patterning across vertebrate taxa will facilitate a deeper understanding of chemical effects on these pathways and interpreting the results of zebrafish behavioral tests.

Likewise, in addition to some of the characteristics that distinguish zebrafish from higher vertebrates, differences in toxicokinetics and metabolic capacities across vertebrates merit discussion. The rate of absorption, distribution, metabolism, and excretion (ADME) is an important parameter for understanding the bioavailability, internal dosimetry, and ultimately the toxicity of a chemical. *In vitro* studies are limited in that ADME cannot be directly observed. The zebrafish provides a functional system for understanding some of the internal dosimetry and dynamics of chemical exposures. They express the full complement of CYPs seen in higher vertebrates and well-conserved Phase 2 enzyme systems such as transferases involved in endogenous and xenobiotic detoxification pathways^{243, 244}. Despite these similarities, the metabolic capacity of embryonic zebrafish in comparison to higher vertebrates continues to be an area in need of study. Another related challenge with zebrafish

HTS assays is being able to extrapolate a nominal concentration spiked in exposure medium to an internal dose in the embryo and a dose relevant for risk analysis. Absent direct measurement at great cost and time, without knowing the embryonic dosimetry kinetics it is difficult to extrapolate results to a mammalian dose for translation to humans and other higher vertebrates. Moreover, metabolic capacity and toxicokinetics in fish can differ from mammals for some chemical classes that may in turn influence toxicity and targets. For instance, oxidative metabolism of PBDE FRs appears to be only a minor metabolic pathway in fish whereas it dominates PBDE metabolism in mammals producing bioactive hydroxy-PBDE (OH-BDE) metabolites ²⁴⁵. Another example of these metabolic differences is observed with some pesticides. Exposures to chlorpyrifos metabolite, chlorpyrifos oxon caused extensive malformations in testing by Truong et al. (2014) whereas chlorpyrifos parent was negative for the same endpoints, thereby reinforcing the importance of metabolic considerations in embryonic screens with zebrafish.

Issues pertaining to differential toxicokinetics across species and life-stages raise questions about the concordance of embryonic zebrafish HTS data with toxicity observations in higher level vertebrates (mice, rats, rabbits). There is a growing body of evidence suggesting high concordance between zebrafish HTS and mammalian toxicity results that is consistent with cross-mammalian comparisons and supportive of predictive-based approaches centered on toxicity pathways ^{246, 247}. However, the physicochemical properties influencing toxicity in embryonic zebrafish HTS assays are less clear. For example, in terms of putative chemical uptake, an important discordant result observed between the ToxCast testing by Padilla et al. (2012) and Truong et al. (2014) pertained to identifying physicochemical properties influencing toxicity. The Padilla study found that toxicity and potency were correlated with chemical hydrophobicity (LogP). As the LogP increased for a chemical so too did its toxicity; however, these positive correlations to LogP were not detected by Truong et al. (2014) that examined a larger set of chemicals and dechorionated embryos prior to exposure, suggesting that uptake, equilibrium partitioning, and ultimately the toxicity are influenced by the chorion. The zebrafish chorion contains pores that are about 0.17 μm^2 that may contribute to size-dependent exclusion of some larger compounds $>3 \text{ kDa}$ ^{130, 248}. In at least one study, consistent with Padilla et al. (2012), chemical toxicity increased in chorionated zebrafish embryos (48 hpf) with chemical lipophilicity, but overall toxicity was greater in embryos that had been dechorionated ²⁴⁹. Moreover, in this same study, while the chorion was reported to not play a role in toxicity for hydrophilic chemicals, exposures among dechorionated embryos caused disturbed swimming in larvae that was not observed among exposed chorionated embryos, suggesting that the chorion offered some functionality. Additional work is needed to understand the role, mechanism, and importance of the chorion in influencing toxicity.

Another area where there continues to be research challenges relates to interpreting the readouts of the rapidly expanding diversity of zebrafish behavioral assays. These assays show great promise for understanding chemical effects on animal behavior for translation to humans and by extension safer chemical design. While the power of PMR behavioral profiling as a predictor of chemical structure toxicity holds promise, additional work would help to continue to expand its use by further defining the specificity of the embryonic PMR mechanism (i.e., stimulation of non-ocular photoreceptors) to development of the nervous

system (i.e., specificity to developmental neurotoxicity). Moreover, questions remain as to whether the embryonic PMR effects are related to neurobehavioral toxicity pathways versus other undetected dysmorphogenesis pathways, or what may be more likely a combination of both neurological and physiological perturbations. Examining chemicals and pharmaceuticals with understood mechanisms and targets with differing potencies would prove beneficial in understanding these relationships. With the large number of transgenic lines, the zebrafish is uniquely suited to characterize how chemical classes and structural attributes target the brain leading to impaired motor and cognitive behaviors.

In addition, the extent to which more recent zebrafish behavioral assays are comparable to mammalian neurobehavioral methods and readouts continues to be a question that will undoubtedly evolve as these test batteries are refined. For instance, unlike rodent and primate behaviors, zebrafish behaviors have not been fully characterized, especially strain-related differences, although progress is being made in defining and cataloging zebrafish behavioral phenotypes²⁵⁰. Another ongoing challenge centers on interpretation and specificity, particularly related to translating and anchoring behavioral phenotypes in zebrafish to specific neurological targets^{251, 252}. These issues also extend to translating behavioral phenotypes measured in zebrafish exposed to chemicals to behavioral responses and targets in higher vertebrates^{250, 253, 254}. There has been work to describe the genes regulating locomotor behavior in larval and embryonic zebrafish²⁵². Ongoing challenges related to linking behavioral phenotypes to specific brain pathologies and neurological mechanisms is not singular to zebrafish, but is relevant for all animals models that are trying to understand how chemical exposures may impair or cause maladaptive motor behaviors and impaired cognitive functioning.

Conclusions

It is clear that the zebrafish confers many advantages in toxicity testing that provide an opportunity to optimize safer chemical design and screen the toxicity of the thousands of chemicals already in use. The rapidly expanding variety of genetic assays, HTS technologies, and behavioral test methods that employ the zebrafish model allow scientists to characterize toxicity across multiple levels of biological organization. In combination, it represents a potential data stream rich in molecular, biochemical, functional, and behavioral information that can be positioned for use in sustainable chemistry efforts. The zebrafish as an *in vivo* biosensor provides an opportunity to posit basic questions about the bioactivity of chemical structures early in product design and R&D to discern physicochemical properties, such as functional groups, chemical classes, and chain-length that may confer less or more activity. Though *in vitro* technologies have important application in toxicity screens, the ability to position zebrafish as a bridge between cell-based tools and other *in vivo* models is an exceptional model attribute that allows for extrapolation of data across physiological targets and vertebrate taxa. The translational advantage of the zebrafish is aided by its shared homology to human orthologs. Capitalizing on these attributes, a number of promising HTS tools measuring bioactivity and behavioral responses are allowing for more automated and rapid ‘safety’ screens of thousands of chemicals. This initial pass into the chemical space does not provide a great deal of insight into underlying toxicity mechanisms, but can be used to identify more sustainable chemistries. With regard to toxicity mechanisms, though, the

zebrafish is an equally important model (e.g., transcriptome profiling, genome editing) catalyzing the shift from empirical tests of chemical effects on apical endpoints (e.g., deformities, survival) to predicting effects on biologically conserved pathways. These tools also provide increasingly meaningful opportunities going forward to characterize the biological effects of chemical mixtures, an area in great need of study. Moreover, these predictive-based approaches are leading to recognition of the integrated connections between human and wildlife health and that the conventional distinctions between human health and ecological risk assessment may not necessarily apply. It is conceivable that the zebrafish could eventually serve as a bridge in future trends to integrate human health and ecological hazard and risk characterizations of chemicals that will be of further use in designing more benign chemicals.

Acknowledgments

The authors would like to thank Paroma Chatterjee, Anna Chlebowsky, and John T. Gamble for generously providing image files of transgenic zebrafish. We also thank Dr. Patience Browne, U.S. EPA, Jane Robbins, U.S. EPA, and Dr. David Dix, U.S. EPA for their review and helpful feedback. Partially supported by EPA STAR grant # R835796.

References

1. Klanova J, Matykiewiczova N, Macka Z, Prosek P, Laska K, Klan P. Environ Pollut. 2008; 152:416–423. [PubMed: 17655988]
2. Beyer A, Mackay D, Matthies M, Wania F, Webster E. Environ Sci Technol. 2000; 34:699–703.
3. Farrington JW, Takada H. Oceanography. 2014; 27:196–213.
4. Grandjean P, Landrigan PJ. Lancet. 2006; 368:2167–2178. [PubMed: 17174709]
5. Trumble SJ, Robinson EM, Noren SR, Usenko S, Davis J, Kanatous SB. Sci Total Environ. 2012; 439:275–283. [PubMed: 23085468]
6. Anastas, PT., Warner, JC. Green Chemistry: Theory and Practice. Oxford University Press; New York, New York: 1998.
7. Manley JB, Anastas PT, Cue BW. Journal of Cleaner Production. 2008; 16:743–750.
8. Collins T. Science. 2001; 291:48–49. [PubMed: 11141549]
9. Matus KJM, Clark WC, Anastas PT, Zimmerman JB. Environ Sci Technol. 2012; 46:10892–10899. [PubMed: 22963612]
10. Schug TT, Abagyan R, Blumberg B, Collins TJ, Crews D, DeFur PL, et al. Green Chem. 2013
11. Dusselier M, Van Wouwe P, Dewaele A, Jacobs PA, Sels BF. Science. 2015; 349:78–80. [PubMed: 26138977]
12. Collins TJ. Accounts of Chemical Research. 2002; 35:782–790. [PubMed: 12234208]
13. Kundu S, Chanda A, Khetan SK, Ryabov AD, Collins TJ. Environ Sci Technol. 2013; 47:5319–5326. [PubMed: 23586823]
14. Shappell NW, Vrabel MA, Madsen PJ, Harrington G, Billey LO, Hakk H, et al. Environ Sci Technol. 2008; 42:1296–1300. [PubMed: 18351108]
15. Tang LL, DeNardo MA, Gayathri C, Gil RR, Kanda R, Collins TJ. Environ Sci Technol. 2016; 50:5261–5268. [PubMed: 27088657]
16. Anastas PT, Lankey RL. Green Chemistry. 2000; 2:289–295.
17. Matus KJM, Clark WC, Anastas PT, Zimmerman JB. Environmental Science & Technology. 2012; 46:10892–10899. [PubMed: 22963612]
18. Matus KJM, Xiao X, Zimmerman JB. J Clean Prod. 2012; 32:193–203.
19. Roschangar F, Sheldon RA, Senanayake CH. Green Chemistry. 2015; 17:752–768.
20. U.S. EPA. Chemical Data Reporting: Chemicals Snapshot Fact Sheet 1. Office of Chemical Safety and Pollution Prevention, U.S. Environmental Protection Agency; Washington DC: 740K13003.

Available online at: http://www2.epa.gov/sites/production/files/2014-11/documents/1st_cdr_basics_factsheet_5_23_2014.pdf [Accessed 23 October 2015]

21. U.S. EPA. Chemical Data Reporting: Chemicals Snapshot Fact Sheet 2. Office of Chemical Safety and Pollution Prevention, U.S. Environmental Protection Agency; Washington DC: 740K13003. Available online at: http://www2.epa.gov/sites/production/files/2014-11/documents/2nd_cdr_snapshot_5_19_14.pdf [Accessed 23 October 2015]
22. U.S. EPA. Pesticide Industry Sales and Usage: 2006 and 2007 Market Estimates. Office of Chemical Safety and Pollution Prevention, U.S. Environmental Protection Agency; Washington DC: Available online at: <http://www2.epa.gov/pesticides/pestsales> [Accessed 23 October 2015]
23. Hengstler JG, Foth H, Kahl R, Kramer PJ, Lilienblum W, Schulz T, et al. Toxicology. 2006; 220:232–239. [PubMed: 16555380]
24. NRC. Toxicity Testing in the 21st Century: A Vision and a Strategy. Available at: http://www.nap.edu/openbook.php?record_id=11970
25. Villeneuve DL, Garcia-Reyero N. Environ Toxicol Chem. 2011; 30:1–8. [PubMed: 21182100]
26. Braunbeck T, Kais B, Lammer E, Otte J, Schneider K, Stengel D, et al. Environ Sci Pollut R. 2015; 22:16247–16261.
27. Holmes AM, Creton S, Chapman K. Toxicology. 2010; 267:14–19. [PubMed: 19903508]
28. Dix DJ, Houck KA, Martin MT, Richard AM, Setzer RW, Kavlock RJ. Toxicol Sci. 2007; 95:5–12. [PubMed: 16963515]
29. Kavlock R, Chandler K, Houck K, Hunter S, Judson R, Kleinstreuer N, et al. Chem Res Toxicol. 2012; 25:1287–1302. [PubMed: 22519603]
30. Padilla S, Corum D, Padnos B, Hunter DL, Beam A, Houck KA, et al. Reprod Toxicol. 2012; 33:174–187. [PubMed: 22182468]
31. Truong L, Reif DM, St Mary L, Geier MC, Truong HD, Tanguay RL. Toxicol Sci. 2014; 137:212–233. [PubMed: 24136191]
32. U.S.EPA. Use of High Throughput Assays and Computational Tools: Endocrine Disruptor Screening Program; Notice of Availability and Opportunity for Comment. Office of Chemical Safety and Pollution Prevention, U.S. Environmental Protection Agency; Washington DC: Jun 19. 2015 80 Fed. Reg. 118 Available online at: <https://www.federalregister.gov/articles/2015/06/19/2015-15182/use-of-high-throughput-assays-and-computational-tools-endocrine-disruptor-screening-program-notice> [Accessed 7 March 2015]
33. OECD. [Accessed 1 October 2016] Workshop Report on OECD Countries Activities Regarding Testing, Assessment, and Management of Endocrine Disruptors. OECD Series on Testing and Assessment, Number 118. Jan 18. 2010 Available online at: [http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO\(2010\)2&doclanguage=en](http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2010)2&doclanguage=en)
34. Fentem J, Chamberlain M, Sangster B. Altern Lab Anim. 2004; 32:617–623.
35. Vandenberg LN, Agerstrand M, Beronius A, Beausoleil C, Bergman A, Bero LA, et al. Environ Health. 2016; 15. [PubMed: 26860262]
36. Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, et al. Nature. 2013; 496:498–503. [PubMed: 23594743]
37. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Dev Dyn. 1995; 203:253–310. [PubMed: 8589427]
38. Peterson RT, Link BA, Dowling JE, Schreiber SL. Proc Natl Acad Sci U S A. 2000; 97:12965–12969. [PubMed: 11087852]
39. Driever W, Solnica-Krezel L, Schier AF, Neuhauss SC, Malicki J, Stemple DL, et al. Development. 1996; 123:37–46. [PubMed: 9007227]
40. Haffter P, Granato M, Brand M, Mullins MC, Hammerschmidt M, Kane DA, et al. Development. 1996; 123:1–36. [PubMed: 9007226]
41. Flicek P, Ahmed I, Amode MR, Barrell D, Beal K, Brent S, et al. Nucleic Acids Res. 2013; 41:D48–D55. [PubMed: 23203987]
42. Vogel G. Science. 2000; 290:1671–1671.
43. Thisse C, Thisse B. Nat Protoc. 2008; 3:59–69. [PubMed: 18193022]
44. Summerton J, Weller D. Antisense Nucleic Acid Drug Dev. 1997; 7:187–195. [PubMed: 9212909]

45. Summerton JE. *Curr Top Med Chem*. 2007; 7:651–660. [PubMed: 17430206]
46. Morcos PA. *Biochem Biophys Res Commun*. 2007; 358:521–527. [PubMed: 17493584]
47. Aranguren XL, Beerens M, Vandevelde W, Dewerchin M, Carmeliet P, Luttun A. *Biochem Biophys Res Commun*. 2011; 410:121–126. [PubMed: 21641336]
48. Chapman AL, Bennett EJ, Ramesh TM, De Vos KJ, Grierson AJ. *PLoS One*. 2013; 8:e67276. [PubMed: 23840650]
49. Kok FO, Shin M, Ni CW, Gupta A, Grosse AS, van Impel A, et al. *Dev Cell*. 2015; 32:97–108. [PubMed: 25533206]
50. Su CY, Kemp HA, Moens CB. *Dev Biol*. 2014; 386:181–190. [PubMed: 24183937]
51. Swift MR, Pham VN, Castranova D, Bell K, Poole RJ, Weinstein BM. *Dev Biol*. 2014; 390:116–125. [PubMed: 24699544]
52. Kim YG, Cha J, Chandrasegaran S. *Proc Natl Acad Sci U S A*. 1996; 93:1156–1160. [PubMed: 8577732]
53. Christian M, Cermak T, Doyle EL, Schmidt C, Zhang F, Hummel A, et al. *Genetics*. 2010; 186:757–761. [PubMed: 20660643]
54. Cade L, Reyon D, Hwang WY, Tsai SQ, Patel S, Khayter C, et al. *Nucleic Acids Res*. 2012; 40:8001–8010. [PubMed: 22684503]
55. Doyon Y, McCammon JM, Miller JC, Faraji F, Ngo C, Katibah GE, et al. *Nature Biotechnol*. 2008; 26:702–708. [PubMed: 18500334]
56. Hruscha A, Krawitz P, Rechenberg A, Heinrich V, Hecht J, Haass C, et al. *Development*. 2013; 140:4982–4987. [PubMed: 24257628]
57. Jao LE, Wente SR, Chen W. *Proc Natl Acad Sci U S A*. 2013; 110:13904–13909. [PubMed: 23918387]
58. Ablain J, Durand EM, Yang S, Zhou Y, Zon LI. *Dev Cell*. 2015; 32:756–764. [PubMed: 25752963]
59. Hwang WY, Fu Y, Reyon D, Maeder ML, Kaini P, Sander JD, et al. *PLoS One*. 2013; 8:e68708. [PubMed: 23874735]
60. Auer TO, Duroure K, De Cian A, Concordet JP, Del Bene F. *Genome Res*. 2014; 24:142–153. [PubMed: 24179142]
61. Kimura Y, Hisano Y, Kawahara A, Higashijima S. *Sci Rep*. 2014; 4:6545. [PubMed: 25293390]
62. Varshney GK, Pei W, LaFave MC, Idol J, Xu L, Gallardo V, et al. *Genome Res*. 2015; 25:1030–1042. [PubMed: 26048245]
63. Howe DG, Bradford YM, Conlin T, Eagle AE, Fashena D, Frazer K, et al. *Nucleic Acids Res*. 2013; 41:D854–860. [PubMed: 23074187]
64. Jones LJ, Norton WHJ. *Behav Brain Res*. 2015; 276:171–180. [PubMed: 24954772]
65. Norton W. *Front Neural Circuit*. 2013; 7 Article 79.
66. Feitsma H, Cuppen E. *Mol Cancer Res*. 2008; 6:685–694. [PubMed: 18505914]
67. Gordon MW, Yan F, Zhong X, Mazumder PB, Xu-Monette ZY, Zou D, et al. *Mol Carcinog*. 2015; 54:1060–1069. [PubMed: 24798859]
68. Nguyen AT, Emelyanov A, Koh CH, Spitsbergen JM, Parinov S, Gong Z. *Dis Model Mech*. 2012; 5:63–72. [PubMed: 21903676]
69. Dalgin G, Prince VE. *Dev Biol*. 2015; 402:81–97. [PubMed: 25797153]
70. Gut P, Baeza-Raja B, Andersson O, Hasenkamp L, Hsiao J, Hesselson D, et al. *Nat Chem Biol*. 2013; 9:97–104. [PubMed: 23201900]
71. Schlegel A, Gut P. *Cell Mol Life Sci*. 2015; 72:2249–2260. [PubMed: 25556679]
72. Arnaout R, Ferrer T, Huisken J, Spitzer K, Stainier DY, Tristani-Firouzi M, et al. *Proc Natl Acad Sci U S A*. 2007; 104:11316–11321. [PubMed: 17592134]
73. Asnani A, Peterson RT. *Dis Model Mech*. 2014; 7:763–767. [PubMed: 24973746]
74. Walcott BP, Peterson RT. *J Cerebr Blood F Met*. 2014; 34:571–577.
75. Bretaud S, Lee S, Guo S. *Neurotoxicol Teratol*. 2004; 26:857–864. [PubMed: 15451049]
76. Da Costa MMJ, Allen CE, Higginbottom A, Ramesh T, Shaw PJ, McDermott CJ. *Dis Model Mech*. 2014; 7:73–81. [PubMed: 24092880]
77. Martin-Jimenez R, Campanella M, Russell C. *Curr Neurol Neurosci*. 2015:15.

78. Preston MA, Macklin WB. *Glia*. 2015; 63:177–193. [PubMed: 25263121]
79. Tropepe V, Sive HL. *Genes Brain Behav*. 2003; 2:268–281. [PubMed: 14606692]
80. Cui, C., Benard, EL., Kanwal, Z., Stockhammer, OW., van der Vaart, M., Zakrzewska, A., et al. *Zebrafish: Disease Models and Chemical Screens*, 3rd Edition. Detrich, HW. Westerfield, M., Zon, LI., editors. Vol. 105. 2011. p. 273–308.
81. Meeker ND, Trede NS. *Dev Comp Immunol*. 2008; 32:745–757. [PubMed: 18222541]
82. Cousin MA, Ebbert JO, Wiinamaki AR, Urban MD, Argue DP, Ekker SC, et al. *Plos One*. 2014; 9:e90467. [PubMed: 24658307]
83. Gerlai R, Lahav M, Guo S, Rosenthal A. *Pharmacol Biochem Be*. 2000; 67:773–782.
84. Ablain J, Zon LI. *Trends Cell Biol*. 2013; 23:584–586. [PubMed: 24275383]
85. Haggard DE, Noyes PD, Waters KM, Tanguay RL. *Toxicol Appl Pharmacol*. 2016; 308:32–45. [PubMed: 27538710]
86. Yang LX, Kemadjou JR, Zinsmeister C, Bauer M, Legradi J, Muller F, et al. *Genome Biol*. 2007; 8:R227. [PubMed: 17961207]
87. Ton C, Stamatiou D, Liew CC. *Physiol Genomics*. 2003; 13:97–106. [PubMed: 12700360]
88. Hochachka PW, Lutz PL. *Comp Biochem Physiol B Biochem Mol Biol*. 2001; 130:435–459. [PubMed: 11691622]
89. van Delft J, Gaj S, Lienhard M, Albrecht MW, Kirpiy A, Brauers K, et al. *Toxicol Sci*. 2012; 130:427–439. [PubMed: 22889811]
90. Wang C, Gong B, Bushel PR, Thierry-Mieg J, Thierry-Mieg D, Xu J, et al. *Nat Biotechnol*. 2014; 32:926–932. [PubMed: 25150839]
91. Harvey SA, Sealy I, Kettleborough R, Fenyés F, White R, Stemple D, et al. *Development*. 2013; 140:2703–2710. [PubMed: 23720042]
92. Aanes H, Winata CL, Lin CH, Chen JP, Srinivasan KG, Lee SG, et al. *Genome Res*. 2011; 21:1328–1338. [PubMed: 21555364]
93. Yang H, Zhou Y, Gu J, Xie S, Xu Y, Zhu G, et al. *PLoS One*. 2013; 8:e64058. [PubMed: 23700457]
94. Li ZH, Xu H, Zheng W, Lam SH, Gong Z. *PLoS One*. 2013; 8:e77292. [PubMed: 24204792]
95. Xu H, Lam SH, Shen Y, Gong Z. *PLoS One*. 2013; 8:e68737. [PubMed: 23922661]
96. Hahn ME, McArthur AG, Karchner SI, Franks DG, Jenny MJ, Timme-Laragy AR, et al. *PLoS One*. 2014; 9:e113158. [PubMed: 25402455]
97. Taft RJ, Pheasant M, Mattick JS. *Bioessays*. 2007; 29:288–299. [PubMed: 17295292]
98. Flynn RA, Chang HY. *Cell Stem Cell*. 2014; 14:752–761. [PubMed: 24905165]
99. Mattick JS. *Nat Rev Genet*. 2004; 5:316–323. [PubMed: 15131654]
100. Tal TL, Tanguay RL. *Neurotoxicol*. 2012; 33:530–544.
101. Zhang L, Li YY, Zeng HC, Wei J, Wan YJ, Chen J, et al. *J Appl Toxicol*. 2011; 31:210–222. [PubMed: 20878907]
102. Vliegenthart AD, Starkey Lewis P, Tucker CS, Del Pozo J, Rider S, Antoine DJ, et al. *Zebrafish*. 2014; 11:219–226. [PubMed: 24625211]
103. Shi X, Yeung LW, Lam PK, Wu RS, Zhou B. *Toxicol Sci*. 2009; 110:334–340. [PubMed: 19474218]
104. Elie MR, Choi J, Nkrumah-Elie YM, Gonnerman GD, Stevens JF, Tanguay RL. *Environ Res*. 2015; 140:502–510. [PubMed: 26001975]
105. Corrales J, Fang X, Thornton C, Mei W, Barbazuk WB, Duke M, et al. *Comp Biochem Physiol C Toxicol Pharmacol*. 2014; 163:37–46. [PubMed: 24576477]
106. Fang X, Corrales J, Thornton C, Scheffler BE, Willett KL. *Comp Biochem Physiol B Biochem Mol Biol*. 2013; 166:99–108. [PubMed: 23876386]
107. Swinney DC, Anthony J. *Nat Rev Drug Discov*. 2011; 10:507–519. [PubMed: 21701501]
108. Armer RE, Morris IA. *Drug News Perspect*. 2004; 17:143–148. [PubMed: 15098069]
109. Rennekamp AJ, Peterson RT. *Curr Opin Chem Biol*. 2015; 24:58–70. [PubMed: 25461724]
110. Hallare A, Nagel K, Kohler HR, Triebkorn R. *Ecotox Environ Safe*. 2006; 63:378–388.

111. Truong L, Mandrell D, Mandrell R, Simonich M, Tanguay RL. *Neurotoxicology*. 2014; 43:134–142. [PubMed: 24674958]
112. Fako VE, Furgeson DY. *Adv Drug Deliv Rev*. 2009; 61:478–486. [PubMed: 19389433]
113. George S, Xia T, Rallo R, Zhao Y, Ji Z, Lin S, et al. *Acs Nano*. 2011; 5:1805–1817. [PubMed: 21323332]
114. Harper SL, Carriere JL, Miller JM, Hutchison JE, Maddux BL, Tanguay RL. *ACS Nano*. 2011; 5:4688–4697. [PubMed: 21609003]
115. Kim KT, Tanguay RL. *Green Chem*. 2013; 15:872–880. [PubMed: 23772181]
116. Lin SJ, Zhao Y, Nel AE, Lin S. *Small*. 2013; 9:1608–1618. [PubMed: 23208995]
117. Truong L, Sali KS, Miller JM, Hutchison JE, Tanguay RL. *Comp Biochem Physiol C Toxicol Pharmacol*. 2012; 155:269–274. [PubMed: 21946249]
118. Noyes PD, Haggard DE, Gonnerman GD, Tanguay RL. *Toxicol Sci*. 2015; 145:177–195. [PubMed: 25711236]
119. Behl M, Hsieh J-H, Shafer TJ, Mundy WR, Rice J, Boyd W, et al. *Neurotoxicol Teratol*. 2015; 49:108–109.
120. Pardo-Martin C, Allalou A, Medina J, Eimon PM, Wahlby C, Yanik MF. *Nat Commun*. 2013; 4:1467. [PubMed: 23403568]
121. Correia T, Lockwood N, Kumar S, Yin J, Ramel MC, Andrews N, et al. *Plos One*. 2015; 10:e0136213. [PubMed: 26308086]
122. Jeanray N, Maree R, Pruvot B, Stern O, Geurts P, Wehenkel L, et al. *Plos One*. 2015; 10:e0116989. [PubMed: 25574849]
123. Ankley GT, Bennett RS, Erickson RJ, Hoff DJ, Hornung MW, Johnson RD, et al. *Environ Toxicol Chem*. 2010; 29:730–741. [PubMed: 20821501]
124. Meek ME, Boobis A, Cote I, Dellarco V, Fotakis G, Munn S, et al. *J Appl Toxicol*. 2014; 34:1–18. [PubMed: 24166207]
125. Nagel R. *Altex*. 2002; 19:38–48. [PubMed: 12096329]
126. Nolen RS. *Javma-J Am Vet Med A*. 2015; 246:12–13.
127. Sneddon LU. *Appl Anim Behav Sci*. 2003; 83:153–162.
128. OECD. [Accessed 20 November 2015] OECD Guidelines for the Testing of Chemicals; fish embryo acute toxicity (FET) test. OECD Guideline 236. Adopted 23 July 2013. Available online at: <http://www.oecd-ilibrary.org/docserver/download/9713161e.pdf?expires=1448064936&id=id&accname=guest&checksum=4561BFD64D5D58A2BBDC9484C133C290>
129. Lammer E, Carr GJ, Wendler K, Rawlings JM, Belanger SE, Braunbeck T. *Comp Biochem Phys C*. 2009; 149:196–209.
130. Scholz S, Fischer S, Gundel U, Kuster E, Luckenbach T, Voelker D. *Environ Sci Pollut R*. 2008; 15:394–404.
131. Voelker D, Vess C, Tillmann M, Nagel R, Otto GW, Geisler R, et al. *Aquat Toxicol*. 2007; 81:355–364. [PubMed: 17292976]
132. Perkins EJ, Ankley GT, Crofton KM, Garcia-Reyero N, LaLone CA, Johnson MS, et al. *Environ Health Perspect*. 2013; 121:1002–1010. [PubMed: 23771518]
133. Hicken CE, Linbo TL, Baldwin DH, Willis ML, Myers MS, Holland L, et al. *Proc Natl Acad Sci U S A*. 2011; 108:7086–7090. [PubMed: 21482755]
134. Hooper MJ, Ankley GT, Cristol DA, Maryoung LA, Noyes PD, Pinkerton KE. *Environ Toxicol Chem*. 2013; 32:32–48. [PubMed: 23136056]
135. Incardona JP, Linbo TL, Scholz NL. *Toxicol Appl Pharm*. 2011; 257:242–249.
136. Billiard SM, Timme-Laragy AR, Wassenberg DM, Cockman C, Di Giulio RT. *Toxicol Sci*. 2006; 92:526–536. [PubMed: 16687390]
137. Bergman A, Heindel JJ, Kasten T, Kidd KA, Jobling S, Neira M, et al. *Environ Health Perspect*. 2013; 121:A104–A106. [PubMed: 23548368]
138. Gore AC, Chappell VA, Fenton SE, Flaws JA, Nadal A, Prins GS, et al. *Endocrine Reviews*. 2015; 36:E1–E150. [PubMed: 26544531]

139. Kortenkamp A, Faust M, Scholze M, Backhaus T. *Environmental Health Perspectives*. 2007; 115:106–114. [PubMed: 18174958]
140. Scholz S, Mayer I. *Mol Cell Endocr*. 2008; 293:57–70.
141. Schug TT, Johnson AF, Birnbaum LS, Colborn T, Guillette LJ, Crews DP, et al. *Molecular Endocrinology*. 2016; 30:833–847. [PubMed: 27477640]
142. Bergman, A.Heindel, JJ.Jobling, S.Kidd, KA., Zoeller, RT., editors. UNEP/WHO. Geneva, Switzerland: 2012.
143. Zoeller RT, Bergman A, Becher G, Bjerregaard P, Bornman R, Brandt I, et al. *Environmental Health*. 2014;13. [PubMed: 24612632]
144. Welshons WV, Thayer KA, Judy BM, Taylor JA, Curran EM, vom Saal FS. *Environ Health Perspect*. 2003; 111:994–1006. [PubMed: 12826473]
145. Vandenberg LN, Colborn T, Hayes TB, Heindel JJ, Jacobs DR, Lee DH, et al. *Endocr Rev*. 2012; 33:378–455. [PubMed: 22419778]
146. Crews D, McLachlan JA. *Endocrinology*. 2006; 147:S4–S10. [PubMed: 16690812]
147. Kubokawa K, Tando Y, Roy S. *Integr Comp Biol*. 2010; 50:53–62. [PubMed: 21558187]
148. McLachlan JA. *Endocrine Reviews*. 2001; 22:319–341. [PubMed: 11399747]
149. Van Der Kraak, G., Zacharewski, T., Janz, DM., Sanders, BM., Gooch, JW. *Principles and Processes for Evaluating Endocrine Disruption in Wildlife*. Kendall, DRRJ.Giesy, JP., Suk, WP., editors. SETAC Press; Pensacola FL: 1998.
150. Bauer MP, Bridgham JT, Langenau DM, Johnson AL, Goetz FW. *Mol Cell Endocrinol*. 2000; 168:119–125. [PubMed: 11064158]
151. Hong Y, Li H, Yuan Y-C, Chen S. *Ann NY Acad Sci*. 2009; 1155:112–120. [PubMed: 19250198]
152. Wilson JY, McArthur AG, Stegeman JJ. *Gen Comp Endocr*. 2005; 140:74–83. [PubMed: 15596073]
153. Ankley GT, Villeneuve DL. *Toxicol Sci*. 2015; 144:259–275. [PubMed: 25564424]
154. Huang Y, Wang XL, Zhang JW, Wu KS. *Reprod Domes Anim*. 2015; 50:1–6.
155. Martinovic-Weigelt D, Wang RL, Villeneuve DL, Bencic DC, Lazorchak J, Ankley GT. *Aquat Toxicol*. 2011; 101:447–458. [PubMed: 21126777]
156. Monteiro MS, Pavlaki M, Faustino A, Rema A, Franchi M, Gediel L, et al. *Journal of Applied Toxicology*. 2015; 35:253–260. [PubMed: 24832558]
157. Sharma P, Grabowski TB, Patino R. *Gen Comp Endocr*. 2016; 226:42–49. [PubMed: 26723187]
158. Wang RL, Bencic D, Biales A, Flick R, Lazorchak J, Villeneuve D, et al. *Bmc Genomics*. 2012;13. [PubMed: 22233093]
159. Zhai WH, Huang ZG, Chen L, Feng C, Li B, Li TS. *Plos One*. 2014;9.
160. Fetter E, Smetanová S, Baldauf L, Lidzba A, Altenburger R, Schüttler A, et al. *Environ Sci Technol*. 2015; 49:11789–11798. [PubMed: 26308493]
161. Schiller V, Wichmann A, Kriehuber R, Schafers C, Fischer R, Fenske M. *Reprod Toxicol*. 2013; 42:210–223. [PubMed: 24051129]
162. Brion F, Le Page Y, Piccini B, Cardoso O, Tong SK, Chung BC, et al. *Plos One*. 2012; 7:e36069. [PubMed: 22586461]
163. Fetter E, Krauss M, Brion F, Kah O, Scholz S, Brack W. *Aquat Toxicol*. 2014; 154:221–229. [PubMed: 24927039]
164. Petersen K, Fetter E, Kah O, Brion F, Scholz S, Tollefsen KE. *Aquat Toxicol*. 2013; 138:88–97. [PubMed: 23721851]
165. Chen H, Yang J, Wang YX, Rang Q, Xu H, Song HY. *Prog Biochem Biphys*. 2006; 33:965–970.
166. Figueiredo MA, Mareco EA, Silva MDP, Marins LF. *Transgen Res*. 2012; 21:457–469.
167. Gorelick DA, Halpern ME. *Endocrinology*. 2011; 152:2690–2703. [PubMed: 21540282]
168. Gorelick DA, Iwanowicz LR, Hung AL, Blazer VS, Halpern ME. *Environ Health Perspect*. 2014; 122:356–362. [PubMed: 24425189]
169. Legler J, Broekhof JLM, Brouwer A, Lanser PH, Murk AJ, Van der Saag PT, et al. *Environmental Science & Technology*. 2000; 34:4439–4444.

170. Ramakrishnan S, Lee W, Navarre S, Kozlowski DJ, Wayne NL. *Gen Comp Endocr*. 2010; 168:401–407. [PubMed: 20515692]
171. Cheng XX, Chen XW, Jin X, He JY, Yin Z. *Toxicol Appl Pharm*. 2014; 278:78–84.
172. Fetter E, Baldauf L, Da Fonte DF, Ortmann J, Scholz S. *Reprod Toxicol*. 2015; 57:10–20. [PubMed: 25962731]
173. Ji C, Jin X, He JY, Yin Z. *Toxicol Appl Pharm*. 2012; 262:149–155.
174. Opitz R, Maquet E, Huiskens J, Antonica F, Trubiroha A, Pottier G, et al. *Dev Biol*. 2012; 372:203–216. [PubMed: 23022354]
175. Terrien X, Fini JB, Demeneix BA, Schramm KW, Prunet P. *Aquatic Toxicology*. 2011; 105:13–20. [PubMed: 21684237]
176. Tiefenbach J, Moll PR, Nelson MR, Hu C, Baev L, Kislinger T, et al. *Plos One*. 2010; 5:e9797. [PubMed: 20339547]
177. Huang HG, Vogel SS, Liu NG, Melton DA, Lin S. *Mol Cell Endocr*. 2001; 177:117–124.
178. Li Z, Wen CM, Peng JR, Korzh V, Gong ZY. *Differentiation*. 2009; 77:128–134. [PubMed: 19281772]
179. Wan HY, Korzh S, Li Z, Mudumana SP, Korzh V, Jiang YJ, et al. *Exp Cell Res*. 2006; 312:1526–1539. [PubMed: 16490192]
180. Krug RG, Poshusta TL, Skuster KJ, Berg MR, Gardner SL, Clark KJ. *Genes Brain Behav*. 2014; 13:478–487. [PubMed: 24679220]
181. Sun LL, Xu W, He JY, Yin Z. *Toxicol Appl Pharm*. 2010; 248:217–225.
182. McEwen B. *Recent Prog Horm Res*. 2002; 57:357–384. [PubMed: 12017552]
183. Andreasen EA, Mathew LK, Lohr CV, Hasson R, Tanguay RL. *Toxicol Sci*. 2007; 95:215–226. [PubMed: 17003102]
184. Cosacak MI, Papadimitriou C, Kizil C. *Biomed Res Int*. 2015; :10.doi: 10.1155/2015/769763
185. Kroehne V, Freudenreich D, Hans S, Kaslin J, Brand M. *Development*. 2011; 138:4831–4841. [PubMed: 22007133]
186. Pasmanik M, Callard GV. *Endocrinol*. 1988; 122:1349–1356.
187. Chiang EFL, Yan YL, Guiguen Y, Postlethwait J, Chung BC. *Mol Biol Evol*. 2001; 18:542–550. [PubMed: 11264405]
188. Sawyer SJ, Gerstner KA, Callard GV. *Gen Comp Endocr*. 2006; 147:108–117. [PubMed: 16458310]
189. Trant JM, Gavasso S, Ackers J, Chung BC, Place AR. *J Exp Zool*. 2001; 290:475–483. [PubMed: 11555854]
190. Menuet A, Pellegrini E, Brion F, Gueguen MM, Anglade I, Pakdel F, et al. *J Comp Neurol*. 2005; 485:304–320. [PubMed: 15803511]
191. Pellegrini E, Mouriec K, Anglade I, Menuet A, Le Page Y, Gueguen MM, et al. *J Comp Neurol*. 2007; 501:150–167. [PubMed: 17206614]
192. Hinfray N, Palluel O, Turies C, Cousin C, Porcher JM, Brion F. *Environ Toxicol*. 2006; 21:332–337. [PubMed: 16841311]
193. Kishida M, McLellan M, Miranda JA, Callard GV. *Comp Biochem Phys B*. 2001; 129:261–268.
194. Le Page Y, Scholze M, Kah O, Pakdel F. *Environ Health Perspect*. 2006; 114:752–758. [PubMed: 16675432]
195. Cachat J, Stewart A, Grossman L, Gaikwad S, Kadri F, Chung KM, et al. *Nature Protocols*. 2010; 5:1786–1799. [PubMed: 21030954]
196. Bencan Z, Sledge D, Levin ED. *Pharmacol Biochem Be*. 2009; 94:75–80.
197. Levin ED, Bencan Z, Cerutti DT. *Physiol Behav*. 2007; 90:54–58. [PubMed: 17049956]
198. Reider M, Connaughton VP. *Behav Neurosci*. 2015; 129:634–642. [PubMed: 26214210]
199. Akingbemi BT, Sottas CM, Koulova AI, Klinefelter GR, Hardy MP. *Endocrinology*. 2004; 145:592–603. [PubMed: 14605012]
200. Vandenberg LN, Maffini MV, Wadia PR, Sonnenschein C, Rubin BS, Soto AM. *Endocrinology*. 2007; 148:116–127. [PubMed: 17023525]

201. Weber DN, Hoffmann RG, Hoke ES, Tanguay RL. *J Toxicol Environ Health A*. 2015; 78:50–66. [PubMed: 25424546]
202. Kokel D, Bryan J, Laggner C, White R, Cheung CYJ, Mateus R, et al. *Nat Chem Biol*. 2010; 6:231–237. [PubMed: 20081854]
203. Kokel D, Dunn TW, Ahrens MB, Alshut R, Cheung CYJ, Saint-Amant L, et al. *J Neurosci*. 2013; 33:3834–3843. [PubMed: 23447595]
204. Reif D, Truong L, Mandrell D, Marvel S, Zhang G, Tanguay R. *Arch Toxicol*. 2015;1–12.
205. Selderslaghs IWT, Hooyberghs J, De Coen W, Witters HE. *Neurotoxicol Teratol*. 2010; 32:460–471. [PubMed: 20211722]
206. Raftery TD, Isales GM, Yozzo KL, Volz DC. *Environ Sci Technol*. 2014; 48:804–810. [PubMed: 24328182]
207. Kamijima M, Casida JE. *Toxicol Appl Pharm*. 2000; 163:188–194.
208. Raftery TD, Volz DC. *Neurotoxicol Teratol*. 2015; 49:10–18. [PubMed: 25733401]
209. Supavilai P, Karobath M. *J Neurochem*. 1981; 36:798–803. [PubMed: 6259289]
210. Burgess HA, Granato M. *J Exp Biol*. 2007; 210:2526–2539. [PubMed: 17601957]
211. Emran F, Rihel J, Dowling JE. *J Visual Exp*. 2008; 20:1–6.
212. Kimmel CB, Patterson J, Kimmel RO. *Dev Psychobiol*. 1974; 7:47–60. [PubMed: 4812270]
213. Sumbre G, Depolavieja GG. *Front Neural Circuit*. 2014; 8:6–9.
214. Ali S, Champagne DL, Spaink HP, Richardson MK. *Birth Defects Res C*. 2011; 93:115–133.
215. MacPhail RC, Brooks J, Hunter DL, Padnos B, Irons TD, Padilla S. *Neurotoxicol*. 2009; 30:52–58.
216. Rihel J, Prober DA, Arvanites A, Lam K, Zimmerman S, Jang S, et al. *Science*. 2010; 327:348–351. [PubMed: 20075256]
217. Steenbergen PJ, Richardson MK, Champagne DL. *Behav Brain Res*. 2011; 222:15–25. [PubMed: 21421013]
218. Carvan MJ, Loucks E, Weber DN, Williams FE. *Neurotoxicol Teratol*. 2004; 26:757–768. [PubMed: 15451040]
219. Irons TD, MacPhail RC, Hunter DL, Padilla S. *Neurotoxicol Teratol*. 2010; 32:84–90. [PubMed: 19465114]
220. Tal TL, Franzosa JA, Tilton SC, Philbrick KA, Iwaniec UT, Turner RT, et al. *FASEB J*. 2012; 26:1452–1461. [PubMed: 22253472]
221. Menelaou E, Udvadia AJ, Tanguay RL, Svoboda KR. *Eur J Neurosci*. 2014; 40:2225–2240. [PubMed: 24738729]
222. Chen J, Huang C, Zheng L, Simonich M, Bai C, Tanguay R, et al. *Neurotoxicol Teratol*. 2011; 33:721–726. [PubMed: 21964161]
223. Saili KS, Corvi MM, Weber DN, Patel AU, Das SR, Przybyla J, et al. *Toxicology*. 2012; 291:83–92. [PubMed: 22108044]
224. Powers CM, Yen J, Linney EA, Seidler FJ, Slotkin TA. *Neurotoxicol Teratol*. 2010; 32:391–397. [PubMed: 20116428]
225. Powers CM, Slotkin TA, Seidler FJ, Badireddy AR, Padilla S. *Neurotoxicol Teratol*. 2011; 33:708–714. [PubMed: 21315816]
226. Chen J, Das SR, La Du J, Corvi MM, Bai C, Chen Y, et al. *Environ Toxicol Chem*. 2013; 32:201–206. [PubMed: 23059794]
227. Wang M, Chen J, Lin K, Chen Y, Hu W, Tanguay RL, et al. *Environ Toxicol Chem*. 2011; 30:2073–2080. [PubMed: 21671259]
228. Chen XJ, Huang CJ, Wang XC, Chen JF, Bai CL, Chen YH, et al. *Aquat Toxicol*. 2012; 120:35–44. [PubMed: 22609740]
229. Dishaw LV, Hunter DL, Padnos B, Padilla S, Stapleton HM. *Toxicol Sci*. 2014; 142:445–454. [PubMed: 25239634]
230. Jarema KA, Hunter DL, Shaffer RM, Behl M, Padilla S. *Neurotoxicol Teratol*. 2015; 52:194–209. [PubMed: 26348672]

231. Crosby EB, Bailey JM, Oliveri AN, Levin ED. *Neurotoxicol Teratol.* 2015; 49:81–90. [PubMed: 25944383]
232. Stanley KA, Curtis LR, Simonich SL, Tanguay RL. *Aquat Toxicol.* 2009; 95:355–361. [PubMed: 19883949]
233. Yang D, Lauridsen H, Buels K, Chi LH, La Du J, Bruun DA, et al. *Toxicol Sci.* 2011; 121:146–159. [PubMed: 21346248]
234. Engeszer RE, Alberici da Barbiano L, Ryan MJ, Parichy DM. *Anim Behav.* 2007; 74:1269–1275. [PubMed: 18978932]
235. Miller N, Gerlai R. *Plos One.* 2012; 7:e48865. [PubMed: 23166599]
236. Gerlach G, Hodgins-Davis A, Avolio C, Schunter C. *P Roy Soc B Biol Sci.* 2008; 275:2165–2170.
237. Mann KD, Turnell ER, Atema J, Gerlach G. *Biol Bull.* 2003; 205:224–225. [PubMed: 14583541]
238. Spence R, Smith C. *Anim Behav.* 2005; 69:1317–1323.
239. Al-Imari L, Gerlai R. *Behav Brain Res.* 2008; 189:216–219. [PubMed: 18243353]
240. Fernandes Y, Tran S, Abraham E, Gerlai R. *Behav Brain Res.* 2014; 265:181–187. [PubMed: 24594368]
241. Sison M, Gerlai R. *Neurobiol Learn Mem.* 2011; 96:230–237. [PubMed: 21596149]
242. Best JD, Berghmans S, Hunt JJ, Clarke SC, Fleming A, Goldsmith P, et al. *Neuropsychopharmacol.* 2008; 33:1206–1215.
243. Goldstone JV, McArthur AG, Kubota A, Zanette J, Parente T, Jonsson ME, et al. *Bmc Genomics.* 2010; 11. [PubMed: 20053297]
244. Vliegenthart AD, Tucker CS, Del Pozo J, Dear JW. *Br J Clin Pharmacol.* 2014; 78:1217–1227. [PubMed: 24773296]
245. Noyes PD, Stapleton HM. *Endocrine Disruptors.* 2014; 2:e29430.
246. Selderslaghs IWT, Van Rompay AR, De Coen W, Witters HE. *Reprod Toxicol.* 2009; 28:308–320. [PubMed: 19447169]
247. Sipes NS, Padilla S, Knudsen TB. *Birth Defects Res C Embryo Today.* 2011; 93:256–267. [PubMed: 21932434]
248. Cheng JP, Flahaut E, Cheng SH. *Environ Toxicol Chem.* 2007; 26:708–716. [PubMed: 17447555]
249. Braunbeck T, Bottcher M, Hollert H, Kosmehl T, Lammer E, Leist E, et al. *Altex Altern Tierexp.* 2005; 22:87–102.
250. Kalueff AV, Gebhardt M, Stewart AM, Cachat JM, Brimmer M, Chawla JS, et al. *Zebrafish.* 2013; 10:70–86. [PubMed: 23590400]
251. Egan RJ, Bergner CL, Hart PC, Cachat JM, Canavello PR, Elegante MF, et al. *Behav Brain Res.* 2009; 205:38–44. [PubMed: 19540270]
252. Granato M, van Eeden FJ, Schach U, Trowe T, Brand M, Furutani-Seiki M, et al. *Development.* 1996; 123:399–413. [PubMed: 9007258]
253. Fetcho JR, McLean DL. *Ann NY Acad Sci.* 2010; 1198:94–104. [PubMed: 20536924]
254. Guo S. *Genes Brain Behav.* 2004; 3:63–74. [PubMed: 15005714]
255. Carlson, BM. *Human embryology and developmental biology.* Elsevier Health Sciences; New York, NY: 2013.
256. O’Rahilly R. *Eur J Obstet Gynecol Reprod Biol.* 1979; 9:273–280. [PubMed: 400868]
257. Witschi, E. *Growth Including Reproduction and Morphological Development.* Altman, PL., Katz, DD., editors. Federation of American Societies for Experimental Biology; Washington, DC: 1962. p. 300-314.

Figure 1. Toxicity pathways leading to perturbed biological responses with chemical exposures. Depending on the potency of the chemical exposure and other biological factors (e.g., life stage, nutritional status, genetics), humans and wildlife may be unable to adapt to toxicant exposures and normal physiological functioning is compromised leading to disease and/or death. (Adapted from NRC 2007).

The Zebrafish Data Stream:

Molecular Snap Shots	Real-time Monitoring of Molecular Events	Real-time Monitoring of Apical Endpoints
Genome	DNA Damage	Developmental Deformities
Epigenome	Oxidative Damage	Neurological Aberrations
Transcriptome	Mitochondrial Dysfunction	Altered Behavioral Responses
Proteome	Gene expression	Disease
Metabolome	Cell Signaling	Reproductive Impairments
Ome interactions (i.e. DNA-Protein)	Cell Migration	Endocrine Disruption
<i>In situ</i> RNA and protein expression	Cell Division	Altered Physiology
	Cell Differentiation	Mortality
	Apoptosis	
Low-Throughput		High-Throughput

Figure 2.

Conceptual diagram of the zebrafish data stream across multiple levels of biological organization.

Figure 3.

Examples of embryonic transgenic zebrafish. (A and B) The *Tg(cyp1a:nls-egfp)* line can be used as a surrogate for AHR activity to identify the target organs of chemically exposed larvae. Embryos were continuously exposed to a chemical starting at 6 hpf and imaged at 48 hpf (A) and 120 hpf (B), with noticeable *cyp1a* expression in the liver at 120 hpf (white arrow). (C and D) *Tg(fli:gfp)* embryos, which expresses GFP in endothelial cells of the entire vasculature, were injected with glioblastoma cells (red) into the brain of 4 dpf larvae (C) and reimaged at 7 dpf (D) to understand the invasion and migration behavior of the brain cancer cells in a vertebrate brain microenvironment. (E–G) Immunohistochemistry was used to determine the *in situ* expression pattern of various genes in the hair cells of the lateral line neuromast of 4 dpf larvae. (E) 2D composite image stained with antibodies targeting otoferlin (blue), acetylated tubulin (green), and maguk (red). (F) 2D composite image stained with antibodies targeting otoferlin (green) and vglut3, a synaptic vesicle marker (red). (G) 3D composite image stained with DAPI and the synaptic protein ribeye (red clusters). While images (E–G) are not from a transgenic line, the images were included to highlight the ability to capture high quality *in situ* expression patterns of genes across development, which is the function of transgenic reporter lines.

Figure 4.

Example of embryonic zebrafish high throughput screening (HTS) platform. Embryos are life staged, screened for viability, and placed into well plates. Chemical exposures typically occur from 6–120 hours post fertilization (hpf). While chemical screens can occur at different life-stages depending on study goals, morphological evaluations and behavioral assays are conducted often during (1) the early pharyngula stage at 24 hpf when the heart is first clearly visible in a distinct pericardial sac and body/tail flexions initiate with development of the sensory-motor system; and (2) free swimming larvae represented by inflation of the swim bladder, largely completed developmental morphogenesis, and rapid growth^{31, 37, 40, 118}.

Author Manuscript

Table 1Comparison of early developmental of human, rat, and zebrafish ^{37, 255–257}

Developmental Stage	Human (Day)	Rat (Day)	Zebrafish (Hour)
Blastula/Blastocyst	4–6	3–5	2–5
Implantation	8–10	6	n/a
Neural Plate Formation	17–19	9.5	10
First Somite	19–21	9–10	10–11
10 Somite Stage	22–23	10–11	14
Neural Tube Formation	22–30	9–12	18–19
First Pharyngeal Arch	22–23	10	24
Initiation of Organogenesis	21	5	10
First Heartbeat	22	10.2	24
Birth/Hatching	253	21	48–72