UNIVERSITY OF CALIFORNIA SAN DIEGO

High-throughput whole-animal screening using freshwater planarian as an alternative model for developmental neurotoxicity

A dissertation submitted in partial satisfaction of the requirements

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in

Bioengineering

by

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The Dissertation of Siqi Zhang is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE DISSERTATION

High-throughput whole-animal screening using freshwater planarian as an alternative model for developmental neurotoxicity

by

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Doctor of Philosophy in Bioengineering

University of California San Diego, 2018

Professor Eva-Maria S. Collins, Chair

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The field of toxicology is under growing pressure to meet the demand of hazard assessment for the increasingly vast number of environmental toxicants. Due to low throughput and high cost, the traditional toxicity testing strategy, using rodent and higher mammalian animal-based models, is unable to adequately meet these competing demands. Hence, there is increasing recognition of the value of transforming toxicity testing to reduce the usage of mammal models and to more efficiently and reliably predict human relevant toxicity. To this end, a battery approach, integrating diverse *in vitro* and alternative animal models, was initiated to complement and accelerate hazard assessment. These alternative models are amenable to

inexpensive, rapid and robust screening. In this dissertation, I introduce the asexual freshwater planarian *Dugesia japonica* as a novel alternative animal model to study developmental neurotoxicity. I developed and expanded a fully automated planarian high-throughput (HTS) screening platform to accomplish rapid screening of multiple morphological and behavioral endpoints. This HTS platform was evaluated for robustness, strengths and weaknesses, using an 87-compound library with known and suspected compounds and a 15-compound flame retardant library. As a unique advantage, the similar size of adult and regenerating planarians allows for direct comparison of two worm types to discern development-specific toxicity from overt systemic toxicity. We show that planarian is a useful model to potentially link toxicity pathways to whole-animal adverse functional outcomes by providing a large repertoire of behavioral endpoints. Comparative analysis of the planarian model with other alternative models, including zebrafish, nematode and *in vitro* cell-based models, provides insight into how different models complement each other in the battery approach. Finally, I discuss our comparative screen of organophosphrous pesticides (OPs) using the expanded platform, to investigate the possible mechanisms of developmental toxicity in OPs. Altogether, by establishing a new alternative animal model this work adds value to the battery approach to accelerate toxicity screening and prioritize toxicants, and provide further insight into the potential mechanisms of OP neurotoxicity.

Chapter 1. Introduction

A call to action in the field of toxicology

Traditionally, toxicology testing has primarily relied on mammalian animal models to provide chemical safety assessments for humans. Such models are considered the current "gold standard" in toxicology (Tsuji and Crofton, 2012), because of their evolutionary proximity to humans. However, the methodology of using mammalian models for toxicology testing is being challenged due to the high cost, low throughput, and ethical concerns. The intrinsic limitations of throughput and cost make it impossible to use this approach to achieve the necessary coverage of the increasingly vast number of environmental toxicants (Burden et al., 2015; Nel et al., 2013). In the United States, the Environmental Protection Agency (EPA) currently lists 68,000 non-confidential substances in the inventory of for the Toxic Substances Control Act (TSCA) with an estimated 700 new compounds introduced to the market each year (Krimsky, 2017). Thus, traditional toxicology testing is unable to fulfill the growing demand to test all existing compounds. Years can pass before a substance is declared toxic or banned. Chlorpyrifos (CPF), a potent organophosphorus pesticide, is a powerful example of the delay of toxicity information. Since its first registration at EPA (www.epa.gov) in 1965, numerous studies, including human studies, have associated high doses with acute toxicity and lower doses with significant adverse developmental effects such as slower motor development and attention problems (Eaton et al., 2008; Rauh et al., 2011; Timofeeva and Levin, 2010). Although residential uses of CPF were phased out in 2000, it is still heavily used in agriculture. Hawaii, the first state in the United States to take action, will ban the use of CPF (bill SB3095) by 2019. However, CPF is still a commonly used pesticide in the rest of the U.S. and a recently proposed federal ban was declined by the current administration in 2017.

Given the growing need for rapid and large-scale toxicity testing, there is increasing recognition in the toxicology community of the urgency of a transformative shift from traditional mammalian models to higher-throughput alternative models. To this end, the "Toxicology Testing in the 21st century" (Tox21) federal initiative was launched in 2008. In the report, *Toxicity Testing in the 21st Century: A Vision and a Strategy*, critical demands for current toxicity testing were pointed out (Krewski et al., 2010), including 1) testing large numbers of existing chemicals lacking basic risk assessment; 2) screening newly developed chemicals; 3) evaluating potential toxicity in the most vulnerable populations (i.e. children); 4) evaluating lower-dose and long-term effects; 5) minimizing animal use; 6) and reducing cost and time. The ongoing goal is to develop and deploy new techniques and alternative models to reduce the usage of mammalian animals for toxicity testing, and to more efficiently and reliably predict potential toxicity in humans (Collins et al., 2008; Krewski et al., 2010). The aim is not to replace mammalian toxicity testing altogether, but to complement and accelerate hazard assessment through a battery approach that integrates various systems (Figure 1.1). Thus, high-throughput protein and cell-based *in vitro* assays and lower organismal models are used to prioritize compounds with potential toxicity on humans for further targeted toxicity assessment in traditional animal models.

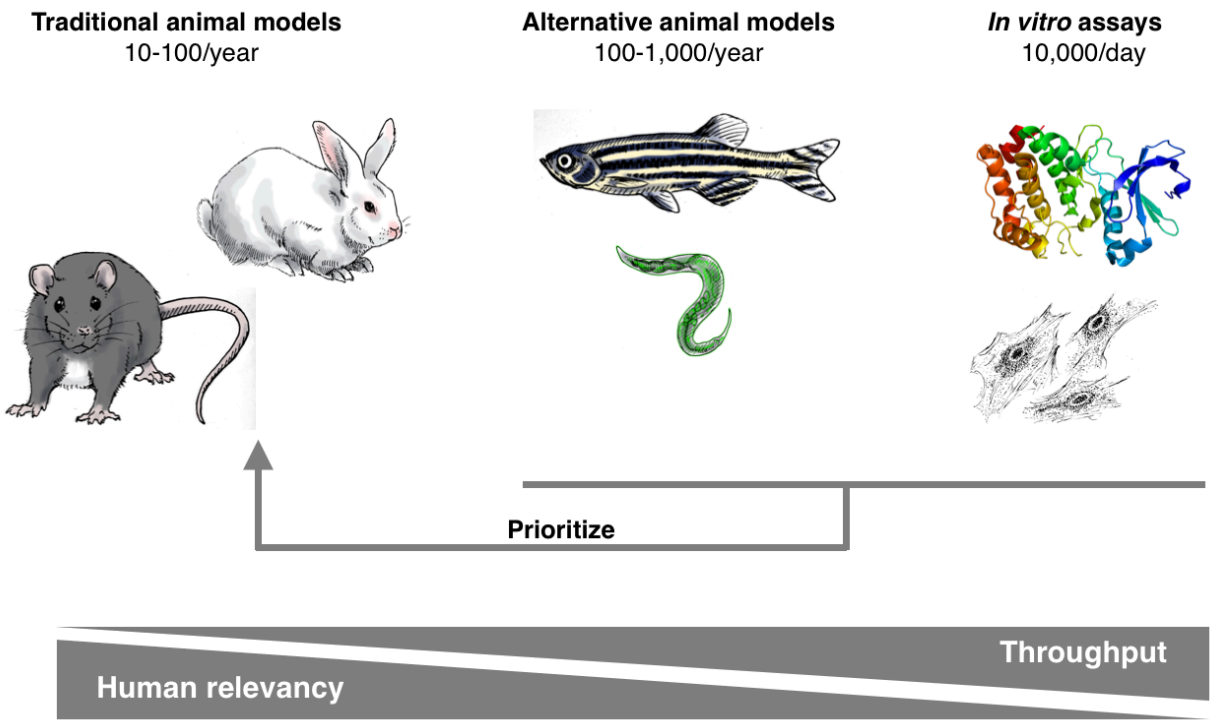

Figure 1.1. Transforming the paradigm of toxicity testing. *In vitro* assays and alternative animal models with high throughput will allow for rapid prioritization of compounds for further targeted testing in traditional animal models and facilitating the prediction of potential toxicity in humans. Drawing courtesy of Rui Wang. The figure was modified from the literature (Collins et al., 2008).

High-throughput *in vitro* assays focus on studying the critical toxicity-relevant pathways on molecular and cellular levels. *In vitro* assays have the advantage of generating large data sets for thousands of chemicals in a fast, low-cost and reliable manner across a broad spectrum of endpoints (Richard et al., 2016; Zhu et al., 2014) (Table 1.1). They provide insight into the toxicity mechanisms at the molecular level. However, it is still difficult to directly translate *in vitro* data (i.e. molecular interactions) to predictions of whole-animal toxicity (i.e. phenotypes). One major gap is the inherent lack of interacting systems in these *in vitro* models, such as interactions between cell types, circulatory systems or metabolic pathways. In addition, although the focus of *in vitro* assays is the key molecular and cellular targets underlying known toxicity pathways, more knowledge is needed to understand the link between disruption of biological pathways and adverse functional impacts on organismal health.

To provide the essential bridge between *in vitro* and mammalian data, multiple alternative animal models amenable to robust whole-animal high-throughput screening (HTS) have emerged as part of the Tox21 initiative, including the nematode *Caenorhabditis elegans* and developing zebrafish. Shared features such as small size, low cost, ease of breeding and maintenance, rapid development, and ease of chemical administration make them excellent organismal models for large-scale toxicology screening (Boyd et al., 2012; Boyd et al., 2015; Horzmann and Freeman, 2018; Truong et al., 2014). Larval nematodes have been used to identify hundreds of compounds with adverse effects on nematode growth and development (Boyd et al., 2015). As a unique strength of nematode model, the genetic mapping of nematodes is well studied, providing the potential to study mechanisms in the living animal. In contrast, as a vertebrate system and due to its optical transparency, developing zebrafish are an excellent

system to study toxic effects on morphological and developmental readouts, and have been used to examine more than 1,000 chemicals in a large-scale screen (Truong et al., 2014).

However, each system has its drawbacks (Table1.1) and no single model is sufficient to predict potential toxicity effects in humans. Even traditional mammalian testing cannot always predict human toxicity well. A meta-study has suggested that only < 50% of toxic effects in humans were correctly predicted from a single rodent model alone, and 63% from non-rodent animal models alone (Olson et al., 2000). Therefore, importantly, a battery-approach using complementary testing systems is necessary. Comparative analyses can produce more weight of evidence and a broader coverage of assays for more reliable prioritization of targets and more relevant predictions of effects on human health. Motivated by this, we have pioneered the freshwater planarian *Dugesia japonica* as one such alternative animal model for developmental neurotoxicology screening and shown it to possess comparable sensitivity to the other, more established alternative models discussed above (Hagstrom et al., 2015).

Planarians as an alternative animal model for developmental neurotoxicology

Freshwater planarians are well known for their remarkable ability to regenerate any missing body structures, including a complete and functional central nervous system (CNS) (Cebrià, 2007; Reddien and Alvarado, 2004). Planarians are one of the simplest animals possessing a CNS, which consists of the anterior brain and two ventral nerve cords that run along the anterior-posterior axis of the animal (Figure 1.2). Although the planarian brain appears structurally simple, it is complex and highly compartmentalized on the cellular level with unipolar, bipolar and multipolar neurons found (Cebrià, 2007). The planarian brain also shares major neurotransmitters with the vertebrate brain, including dopamine, serotonin, GABA, and acetylcholine. (Cebrià, 2007; Cebrià et al., 2002; Umesono et al., 2011). Additionally, >95% of planarian nervous system-related genes are shared in fruit flies, nematodes and humans, suggesting a high level of conservation (Mineta et al., 2003). All these features suggest that despite the simple morphology, the planarian brain possesses the complexity at the cellular and molecular level to be relevant to model vertebrate neurodevelopment (Mineta et al., 2003; Umesono et al., 2011). In asexual planarians, which only reproduce by binary fission, head regeneration is the sole mechanism for neurodevelopment and can be induced “at will” through amputation.

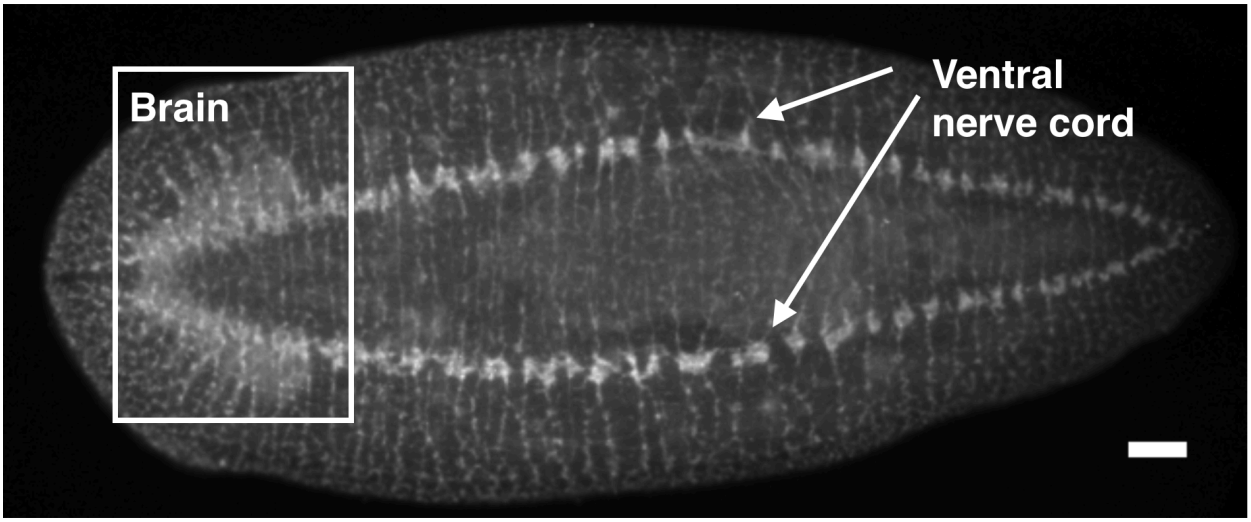

Figure 1.2. Anti-synapsin staining of the nervous system in *Dugesia japonica*. Planarian central nervous system composed of brain (box) in the head region and two ventral nerve cords (arrows). Image courtesy of Danielle Hagstrom. Scale bar: 100μm.

Planarians possess a large repertoire of behavioral and morphological endpoints which can be quantified to assess different aspects of regeneration and neural functions. More importantly, as a strength of the planarian system, these morphological and behavioral effects can be connected with molecular and cellular effects through mechanistic studies because of the intermediate complexity of its CNS (Hagstrom et al., 2016). Different neuronal populations and pathways involved in different behaviors were characterized and most of the neuronal populations and pathways are conserved in the vertebrate brain, (Cochet-Escartin et al., 2015; Inoue et al., 2014; Inoue et al., 2015; Nishimura et al., 2008). For example, it has been demonstrated that GABAergic neurons are necessary for phototaxis behavior (negative response to light) in planarians (Nishimura et al., 2008).

Because of their ability of regeneration, relevancy to the vertebrate brain and the large array of behavioral and morphological endpoints, planarians have been used previously in studies of developmental neurotoxicity (Lowe et al., 2015; Van Huizen et al., 2017; Zhang et al., 2013). These previous studies have focused on the toxicity mechanism of small numbers of compounds, and indicated that planarians are sensitive to certain toxicants during development. However, they did not meet the challenges of 21st century toxicity testing due to throughput and limited coverage of endpoints and chemicals. To meet these challenges and achieve the necessary throughput, automation of experiments and data analysis are indispensable. Robustness also needs to be achieved by automating more endpoints and screening more replicates to make sure data are unbiased and reliable. Moreover, since various research groups have used different planarian species and different experimental methods (i.e. exposure time and methods of behavioral assay), it is difficult to compare results across studies even on the same chemical. Thus, a unification of methodology is required for toxicity screening. Together, these

considerations emphasize the necessity of a standardized and systematic screening approach to efficiently assess chemicals in large scale in planarians.

Therefore, in this work, we established an automated HTS alternative animal platform using the freshwater planarian, *D. japonica*. As a unique advantage of this system, the similar size of adult and regenerating planarian allows us to compare both worm types in parallel in the same assays to discern development-specific toxicity. Our system provides various morphological and behavioral endpoints, allowing the ability to differentiate function-specific toxicity from overt systemic toxicity. Table 1.1 summarizes and compares the planarian system to the other popular alternative systems, listing each system's unique strengths and limitations, which are complemented by the other systems. This is why a battery testing approach is the most desirable strategy.

Table 1.1. Critical advantages and limitations of existing alternative *in vivo* and protein- and cell- based *in vitro* models.

	Advantages	Limitations
In vitro	• High throughput • Low cost • Standardized and controlled conditions • Require little volume of test compound • Free of ethical concerns	• Artificial culture conditions • Lack of xenobiotic metabolism • Lack of interactions between different cell types • Difficult to extrapolate perturbed pathways or biomarkers to whole-animal adverse effects
Zebrafish	• High throughput animal model • Whole-animal system, vertebrate • Low cost • Small size • External fertilization • Transparent embryo • Multiple morphological readouts	• Difficult to control dose absorption • Limited study of xenobiotic metabolism • Potential solubility issues • Potential ethical issues
Nematode	• High throughput animal model • Whole-animal system, invertebrate • Low cost • Small size • Free of ethical concerns • Genetically tractable • Have behavioral response to stimuli	• Difficult to control dose absorption • Lack of structure complexity • Limited substance absorption due to its tough cuticle • Strict requirement of culture
Planarian	• High throughput animal model • Whole-animal system, invertebrate • Low cost • Small size • Free of ethical concerns • Multiple quantifiable behaviors • In-parallel comparison of adult and developing animal in same assays	• Difficult to control dose absorption • Limited study of xenobiotic metabolism • Potential solubility issues • Clonal animals, no genetic diversity

Summary

In this chapter, I introduced the current status and challenges for toxicology and argued for an urgent need of a transformative paradigm shift in toxicity testing. Therefore, a battery-approach using multiple complementary screening models was prompted in toxicology to accelerate chemical assessment, and provide more accurate prediction of human-relevant toxicity. I have introduced the more established alternative toxicology models, including *in vitro* models and alternative animal models (zebrafish and nematode). I have reviewed the advantages and unique features of using the freshwater planarians *D. japonica* as an organism to study developmental neurotoxicity and compared them to those of other alternative models. In chapter 2, I will discuss the results of a proof-of-concept screen to evaluate the potential of *D. japonica* as a complementary alternative for developmental neurotoxicology screening models. In chapter 3, I will introduce the automated planarian high-throughput screening platform we established, and evaluate the strengths and weaknesses of the planarian system using an 87-compound library. In chapter 4, I will provide a comparative analysis of the planarian system with a developing zebrafish system using the same 87-compound library, and discuss the value of using two systems for chemical prioritization. In chapter 5, I will further evaluate the robustness of the planarian screening system using a library of 15 flame retardants and compare our results with published data from other alternative models and mammalian data to evaluate the concordance and sensitivity of our system. In Chapter 6, I will discuss a comparative screen to investigate the mechanism by which organophosphorus pesticides may cause developmental neurotoxicity. In Chapter 7, I will summarize the importance of the work and propose future directions.

References

Boyd, W. A., Smith, M. V and Freedman, J. H. (2012). Caenorhabditis elegans as a model in developmental toxicology. *Methods Mol. Biol.* **889**, 15–24.

Boyd, W. A., Smith, M. V., Co, C. A., Pirone, J. R., Rice, J. R., Shockley, K. R. and Freedman, J. H. (2015). Developmental Effects of the ToxCast^{TM} Phase I and Phase II Chemicals in Caenorhabditis elegans and Corresponding Responses in Zebrafish, Rats, and Rabbits. *Environ. Health Perspect.* **124**, 586–93.

Burden, N., Sewell, F. and Chapman, K. (2015). Testing Chemical Safety: What Is Needed to Ensure the Widespread Application of Non-animal Approaches? *PLoS Biol.* **13**, e1002156.

Cebrià, F. (2007). Regenerating the central nervous system: how easy for planarians! *Dev. Genes Evol.* **217**, 733–48.

Cebrià, F., Kudome, T., Nakazawa, M., Mineta, K., Ikeo, K., Gojobori, T. and Agata, K. (2002). The expression of neural-specific genes reveals the structural and molecular complexity of the planarian central nervous system. *Mech. Dev.* **116**, 199–204.

Cochet-Escartin, O., Mickolajczyk, K. J. and Collins, E.-M. S. (2015). Scrunching: a novel escape gait in planarians. *Phys. Biol.* **12**, 56010.

Collins, F. S., Gray, G. M. and Bucher, J. R. (2008). Toxicology. Transforming environmental health protection. *Science* **319**, 906–7.

Eaton, D. L., Daroff, R. B., Autrup, H., Bridges, J., Buffler, P., Costa, L. G., Coyle, J., McKhann, G., Mobley, W. C., Nadel, L. and Neubert, D. (2008). Review of the Toxicology of Chlorpyrifos With an Emphasis on Human Exposure and Neurodevelopment. *Crit. Rev. Toxicol.* **38**, 1–125.

Hagstrom, D., Cochet-Escartin, O., Zhang, S., Khuu, C. and Collins, E. M. S. (2015). Freshwater planarians as an alternative animal model for neurotoxicology. *Toxicol. Sci.* **147**, 270–285.

Hagstrom, D., Cochet-Escartin, O. and Collins, E.-M. S. (2016). Planarian brain regeneration as a model system for developmental neurotoxicology. *Regeneration* **3**, 65–77.

Horzmann, K. A. and Freeman, J. L. (2018). Making Waves: New Developments in Toxicology With the Zebrafish. *Toxicol. Sci.* **163**, 5–12.

Inoue, T., Yamashita, T. and Agata, K. (2014). Thermosensory signaling by TRPM is processed by brain serotonergic neurons to produce planarian thermotaxis. *J. Neurosci.* **34**, 15701–14.

Inoue, T., Hoshino, H., Yamashita, T., Shimoyama, S., Agata, K. (2015). Planarian shows decision-making behavior in response to multiple stimuli by integrative brain function.

Zool. Lett. **1**, 7.

Krewski, D., Acosta, D., Andersen, M., Anderson, H., Bailar, J. C., Boekelheide, K., Brent, R., Charnley, G., Cheung, V. G., Green, S. and Kelsey, K.T. (2010). Toxicity Testing in the 21st Century: A Vision and a Strategy. *J. Toxicol. Environ. Heal. Part B* **13**, 51–138.

Krimsky, S. (2017). The unsteady state and inertia of chemical regulation under the US Toxic Substances Control Act. *PLoS Biol.* **15**, e2002404.

Lowe, J. R., Mahool, T. D. and Staehle, M. M. (2015). Ethanol exposure induces a delay in the reacquisition of function during head regeneration in Schmidtea mediterranea. *Neurotoxicol. Teratol.* **48**, 28–32.

Mineta, K., Nakazawa, M., Cebria, F., Ikeo, K., Agata, K. and Gojobori, T. (2003). Origin and evolutionary process of the CNS elucidated by comparative genomics analysis of planarian ESTs. *Proc. Natl. Acad. Sci.* **100**, 7666–7671.

Nel, A., Xia, T., Meng, H., Wang, X., Lin, S., Ji, Z. and Zhang, H. (2013). Nanomaterial toxicity testing in the 21st century: use of a predictive toxicological approach and high-throughput screening. *Acc. Chem. Res.* **46**, 607–21.

Nishimura, K., Kitamura, Y., Umesono, Y., Takeuchi, K., Takata, K., Taniguchi, T. and Agata, K. (2008). Identification of glutamic acid decarboxylase gene and distribution of GABAergic nervous system in the planarian Dugesia japonica. *Neuroscience* **153**, 1103–1114.

Olson, H., Betton, G., Robinson, D., Thomas, K., Monro, A., Kolaja, G., Lilly, P., Sanders, J., Sipes, G., Bracken, W. and Dorato, M. (2000). Concordance of the Toxicity of Pharmaceuticals in Humans and in Animals. *Regul. Toxicol. Pharmacol.* **32**, 56–67.

Rauh, V., Arunajadai, S., Horton, M., Perera, F., Hoepner, L., Barr, D. B. and Whyatt, R. (2011). Seven-Year Neurodevelopmental Scores and Prenatal Exposure to Chlorpyrifos, a Common Agricultural Pesticide. *Environ. Health Perspect.* **119**, 1196–1201.

Reddien, P. W. and Alvarado, A. S. (2004). FUNDAMENTALS OF PLANARIAN REGENERATION. *Annu. Rev. Cell Dev. Biol.* **20**, 725–757.

Richard, A. M., Judson, R. S., Houck, K. A., Grulke, C. M., Volarath, P., Thillainadarajah, I., Yang, C., Rathman, J., Martin, M. T., Wambaugh, J. F. and Knudsen, T.B. (2016). ToxCast Chemical Landscape: Paving the Road to 21st Century Toxicology. *Chem. Res. Toxicol.* **29**, 1225–1251.

Timofeeva, O. A. and Levin, E. D. (2010). Lasting Behavioral Consequences of Organophosphate Pesticide Exposure During Development. In *Hayes' Handbook of Pesticide Toxicology*, pp. 837–846. Elsevier.

Truong, L., Reif, D. M., St Mary, L., Geier, M. C., Truong, H. D. and Tanguay, R. L. (2014). Multidimensional in vivo hazard assessment using zebrafish. *Toxicol. Sci.* **137**, 212–33.

Tsuji, R. and Crofton, K. M. (2012). Developmental neurotoxicity guideline study: Issues with methodology, evaluation and regulation*. *Congenit. Anom. (Kyoto)*. **52**, 122–128.

Umesono, Y., Tasaki, J., Nishimura, K., Inoue, T. and Agata, K. (2011). Regeneration in an evolutionarily primitive brain - the planarian Dugesia japonica model. *Eur. J. Neurosci.* **34**, 863–869.

Van Huizen, A. V., Tseng, A.-S. and Beane, W. S. (2017). Methylisothiazolinone toxicity and inhibition of wound healing and regeneration in planaria. *Aquat. Toxicol.* **191**, 226–235.

Zhang, J., Yuan, Z., Zheng, M., Sun, Y., Wang, Y. and Yang, S. (2013). Effects of *N* , *N* -dimethylformamide on behaviour and regeneration of planarian *Dugesia japonica*. *Toxicol. Ind. Health* **29**, 753–760.

Zhu, H., Zhang, J., Kim, M. T., Boison, A., Sedykh, A. and Moran, K. (2014). Big data in chemical toxicity research: the use of high-throughput screening assays to identify potential toxicants. *Chem. Res. Toxicol.* **27**, 1643–51.

Chapter 2. Freshwater planarians as an alternative animal model for neurotoxicology

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Supplementary data is available online (https://academic.oup.com/toxsci/article/147/1/270/1642148#supplementary-data).

Abstract

Traditional toxicology testing has relied on low-throughput, expensive mammalian studies; however, timely testing of the large number of environmental toxicants requires new in vitro and in vivo platforms for inexpensive medium to high-throughput screening. Herein, we describe the suitability of the asexual freshwater planarian Dugesia japonica as a new animal model for the study of developmental neurotoxicology. As these asexual animals reproduce by binary fission, followed by regeneration of missing body structures within approximately one week, development and regeneration occur through similar processes allowing us to induce neurodevelopment "at will" through amputation. This short time-scale and the comparable sizes of full and regenerating animals enable parallel experiments in adults and developing worms to determine development-specific aspects of toxicity. Because the planarian brain, despite its simplicity, is structurally and molecularly similar to the mammalian brain, we are able to ascertain neurodevelopmental toxicity which is relevant to humans. As a proof of concept, we developed a five-step semi-automatic screening platform to characterize the toxicity of nine known neurotoxicants (consisting of common solvents, pesticides, and detergents) and a neutral agent, glucose, and quantified effects on viability, stimulated and unstimulated behavior, regeneration, and brain structure. Comparisons of our findings with other alternative toxicology animal models, namely zebrafish larvae and nematodes, demonstrated that planarians are comparably sensitive to the tested chemicals. Additionally, we found that certain compounds induced adverse effects specifically in developing animals. We thus conclude that planarians offer new, complementary opportunities for developmental neurotoxicology animal models.

Introduction

The "Tox21" initiative (http://epa.gov/ncct/Tox21/), a multi-agency partnership was launched in 2008 to establish a new area in toxicology testing, away from low-throughput, high-cost mammalian models, toward in vitro and alternative non-mammalian animal systems amenable to low-cost, high-throughput screens (HTS) (Vliet, 2011). To achieve this, the ToxCast program (http://www.epa.gov/ncct/toxcast/) was launched, using a large-scale in vitro HTS robotic approach to evaluate thousands of chemicals for a variety of potential molecular and cellular toxicity effects (Judson et al., 2010). However, the inherently artificial environment and lack of biological complexity in in vitro HTS makes them difficult to directly connect with organism-level toxicity (Knight et al., 2009). Therefore, as the second component of the Tox21 agenda, medium-throughput-screening (MTS) animal models were introduced to complement HTS assays (Collins et al., 2008). Because each animal model has specific strengths and weaknesses, in terms of throughput, cost, and homology to humans, any one system is insufficient to cover all aspects of toxicity in humans, making comparative analyses across diverse animals important for the proper prioritization of toxicants for further study and development of human exposure guidelines.

In this study, we establish the suitability of freshwater planarians, famous for their regenerative capabilities due to a large population of adult pluripotent stem cells (Cebrià, 2007; Reddien and Sánchez Alvarado, 2004; Rink, 2013; Scimone et al., 2014; Wagner et al., 2011), as a new model for MTS toxicology studies. In terms of organismal complexity, planarians occupy an intermediate position, between the newly developed alternative toxicology animal models zebrafish and nematodes (Boyd et al., 2012; Peterson et al., 2008; Selderslaghs et al., 2009; Sipes et al., 2011; Truong et al., 2014) and possess unique features that make them especially well-

suited for developmental neurotoxicology. Like zebrafish and nematodes, freshwater planarians are small, inexpensive and easy to breed, sensitive to chemicals in the water, and develop quickly (in approximately 1 week). For the asexual Dugesia japonica species used in this study, development and regeneration are similar processes as these animals reproduce by transverse fission creating a head and a tail piece, each subsequently regenerating all missing body structures (Sakurai et al., 2012). We can thus induce development "at will" by amputation in a purely clonal population.

What renders freshwater planarians unique and particularly well-suited for developmental neurotoxicology is our ability to simultaneously study genetically identical adult and developing animals, allowing us to directly compare the effect of potential toxicants on the adult and developing brain, without possible complications from the variability of genetic factors. In addition, the planarian nervous system, consisting of a bi-lobed cephalic ganglion (brain) and ventral nerve cords, is much more complex than that of nematodes, but simpler than that of zebrafish. It remains tractable on the cellular level (~10000 neurons) while having sufficient complexity and homology, sharing the same neuronal subpopulations and neurotransmitters as the mammalian brain, to be relevant to human studies (Buttarelli et al., 2008; Cebrià, 2007; Cebrià et al., 2002). In fact, the planarian brain is thought to be more similar to the vertebrate brain than to other invertebrate brains in terms of structure and function (Buttarelli et al., 2008). Most notably, 95% of nervous system related genes in Dugesia japonica have homologs in humans (Mineta et al., 2003). Thus, by studying planarian brain development, we can gain insight into key mechanisms for human brain development.

As a result, various species of freshwater planarians have previously been used for pharmacological and toxicological studies (Lowe et al., 2015; Pagán et al., 2006; Stevens et al.,

2014; Talbot and Schötz, 2011). These studies, albeit primarily focused on a single compound, low-throughput, and largely qualitative in nature, demonstrated that planarians are highly sensitive to certain chemicals and that toxicity can be assessed via behavioral and morphological readouts.

In this study, we evaluated the potential of the planarian system as a new model for MTS toxicology studies by studying nine known neurotoxicants and the neutral compound, glucose. Using a proof-of-concept screen, we determined and characterized, for these compounds, the lethal dose, systemic and behavioral effects, and neurotoxicity, resulting from exposure in adult and developing animals. We show that D. japonica has comparable sensitivity to other model systems, as evaluated by a quantitative comparison of our data with data from zebrafish and nematodes. Furthermore, by studying full and developing animals simultaneously, we detected toxicity specific to the developing brain. Based on these results, we conclude that planarians are well-suited for screening potential developmental neurotoxicants and allow for the addition of a new alternative animal model to the field of neurotoxicology.

Material and methods

Test animals: Freshwater planarians of the species Dugesia japonica were used for all tests. Planarians were stored in 1x planarian water (Cebrià and Newmark, 2005) in Tupperware containers at 20°C in a Panasonic refrigerated incubator in the dark. Animals were fed organic beef liver once a week and cleaned twice a week when not used for experiments (Dunkel et al., 2011). Test animals were randomly selected from a healthy population. For all experiments, only fully regenerated worms which had not been fed within one week and which were found gliding normally in the container were used. Worms were manually selected to fall within a certain range of sizes and we found them, after automated size measurement, to be 3.4mm +/- 0.7mm (mean +/- SD) in length. To study regenerating animals, on day 1, intact worms were amputated with an ethanol-sterilized razor blade no more than 3 hours before an assay was started.

Test compounds: The following were tested and reconstituted according to manufacturer guidelines as described below: dimethyl sulfoxide (DMSO, Sigma Aldrich, D2650), permethrin (Sigma Aldrich, 44-2748), chlorpyrifos (Fluka Analytical, 45395), dichlorvos (Chem Service, N-11675), ethanol (Roptec, V1001), methanol (Fisher Scientific, A454), TritonX-100 (Alfa Aesar, A16046), sodium dodecyl sulfate (SDS, Promega, H5113), acrylamide (Tokyo Chemical Industry, A1132), and D-glucose (Sigma-Aldrich, D9434). All solutions were prepared in 1x planarian water. Chemicals which were soluble in water, i.e. DMSO, dichlorvos, ethanol, methanol, TritonX-100, SDS, acrylamide, and D-glucose, were added directly to planarian water to obtain the desired concentrations. Stocks of 500mM chlorpyrifos and 100mM permethrin were prepared in 100% DMSO such that, in the final working solutions, the DMSO concentration did not exceed 0.1%. All solutions were checked with a pH-meter and were found to fall within a reasonable range (pH 7.39 – 7.75). Working solutions were stored at room temperature. DMSO,

acrylamide, and permethrin solutions were stored in the dark. To mitigate diminishing effects due to evaporation, all ethanol solutions were replaced daily. Table 2.1 summarizes tested chemicals and concentrations.

Table 2.1. Chemicals and concentration ranges tested.

Compound	CAS	Source	Purity (%)	Concentration range tested
Acrylamide	79-06-1	Tokyo Chemical Industry	98.0	10 μM to 100 mM
Chlorpyrifos	2921-88-2	Fluka Analytical	99.7	0.1–500 μM
Dichlorvos	62-73-7	Chem Service	97.8	10 nM to 8 μM
DMSO	67-68-5	Sigma Aldrich	99.7	0.05–15%
Ethanol	64-17-5	Roptec	100	0.01–15%
Glucose	50-99-7	Sigma-Aldrich	99.5	55 μM to 550 mM
Methanol	67-56-1	Fisher Scientific	99.9	0.5–7%
Permethrin	52645-53-1	Sigma-Aldrich	N/A	10–1000 μM
SDS	151-21-3	Promega	99.5	0.2–6 mg/l
TritonX-100	9002-93-1	Alfa Aesar	N/A	5–50 mg/l

Lethality assay: The first step in determining the toxicity of a compound was a broad range screen on its effect on planarian health and regeneration. Small planarians were selected as described above and distributed into a 48-well plate (Falcon, 353078) such that each well contained one worm. Each row was filled with half full and half recently amputated (less than 3h) animals. Once a plate was completely filled with worms, the planarian water was removed and 200µL of the appropriate chemical solution was added to each well. For each concentration of a chemical, at least two independent experiments with 8 full worms and 8 regenerating worms were performed as biological replicates, thus at least 16 full and regenerating animals were assayed for each condition.

Animals were stored in the plate for 15 days at room temperature in the dark. Worms which did not move even after gentle prodding or agitation of the water were considered dead. Deaths were manually inspected and tallied in Microsoft Excel. The resulting data was manually imported into MATLAB (Mathworks) for plotting and analysis. The fraction of dead worms as a function of concentration at days 2, 4, 8, and 15 was plotted and fitted using (Selderslaghs et al., 2009):

$$y = \left(\frac{1}{1 + 10^{(\log LC_{50} - x) \times \text{Hill slope}}}\right)$$

with y the fraction of dead individuals, x the logarithm of the chemical concentration to obtain the LC_{50} and Hill slope is the slope factor of the dose-response curve. The two asymptotes of the original Hill equation were forced to be 0 and 1 since most of our ranges were sufficient to cover these two asymptotes. In one instance, we did not calculate a LC_{50} value due to lack of death and in two instances with insufficient data to cover these asymptotes this choice lead to an increased uncertainty in the LC_{50} measurements.

Unstimulated behavioral assays: For each toxicant concentration tested, 24 planarians

were placed in two 12-well plates (Falcon, 353043), with a single worm placed in each well, and their locomotion was determined using automated center-of-mass (COM) tracking (Figure S1A-B). Once both plates were filled, the planarian water was removed and 500µl of the appropriate concentration of chemical was added to each well. For evaluation of acute toxicity, plates were imaged within five minutes of adding the chemical. These same worms were also imaged after eight days of exposure. To assay the locomotion of regenerating worms, for each concentration, 24 planarians were amputated using an ethanol-sterilized razor blade and immediately stored in a 48-well plate, with a single worm in each well containing 200µl of the appropriate chemical. On day 8, the regenerating worms were transferred to two 12-well plates containing a single worm and 500µl of the respective chemical per well. Regenerating worms were imaged eight and fifteen days after amputation and chemical exposure. Except during imaging, the plates were stored at 20°C in the dark.

The imaging system consisted of a ring stand with a CCD camera (PointGrey Flea3 1.3MP Mono USB 3.0) equipped with a 16mm lens (Tamron M118FM16 Megapixel Fixed-focal Industrial Lens). The plates were illuminated from below using a cold LED panel (Amazon.com). Image acquisition was controlled through a custom LabVIEW (National Instruments) script. The two plates were imaged at 5 frames per second (fps) for 10 minutes, following our previously established protocol for characterizing behavioral phenotypes in response to drug exposure (Talbot and Schötz, 2011). Image analysis was performed using custom made scripts in MATLAB. An average intensity projection image was first generated from the entire movie and subtracted from each picture in the stack. The resulting images were thresholded to obtain the worm's outline and each worm was automatically assigned a well number while its center of mass, length, and area were recorded. Worm tracks were sometimes

truncated when worms were lost at the well edges. Only tracks longer than 2s were analyzed. Instantaneous speeds (in mm/s) were calculated for all tracks at 2s intervals to improve the signal to noise ratio (Talbot and Schötz, 2011).

We distinguished between three different behaviors: resting, swimming, and gliding. A speed below 0.3mm/s was considered as the worm's resting or wiggling speed. To distinguish swimming from gliding, we defined a dynamic cutoff as follows: the speed distribution of the entire population of 24 worms was computed and fitted by the sum of two Gaussians and a constant value (Figure S1C) according to:

$$a_1 e^{\frac{-(x-\mu_1)^2}{{\sigma_1}^2}} + a_2 e^{\frac{-(x-\mu_2)^2}{{\sigma_2}^2}} + c$$

The fit was performed using the built-in MATLAB fit function and non-linear least square method. The fit output was shown graphically on top of the raw data. In case of poor fit results, the user could manually determine the relevant parameters instead. Worms were considered as gliding at any time point for which the speed was larger than -1.5 , a value was adapted by hand to represent the behavior of control populations. The worms were declared swimming at time points for which the speed was between the absolute resting cutoff and this dynamic gliding cutoff (Figure S1C).

From this population level classification, each worm was assigned a fraction of time spent in each of the three behaviors for all time points tracked. To remove bias due to differences in worm size, we scaled the animal's speed by its aspect ratio, calculated as the ratio of the worm's length squared to the worm area,$/$or$/$, to reduce noise in the measurement. Based on control populations and the MATLAB built-in power law fit tool, we found that the gliding speed scaled with the power 2/3 of this aspect ratio (Figure S1D). We therefore defined a scaled gliding speed as the absolute gliding speed divided by that measurement. All measurements were

averaged over the entire population (n=24) and error bars were calculated as the standard error of the mean. Of note, the contribution of each worm to the mean was not weighed by the time for which it was tracked, thus treating all worms equal.

Thermotaxis assay: For every tested concentration, twenty worms were amputated and allowed to regenerate for 15 days in the respective concentration of chemical. After this period, the regenerating worms were placed in a single 100mm Petri dish filled with 20mL of planarian water. The dish was placed atop a kimwipe on top of a custom 10.5cm wide circular Peltier cooler with a central 3cm wide square cold plate surrounded by a circular heat sink. This cooler was powered by a DC regulated power supply (BK Precision) set to 5V. During the assay, the temperature was initially homogenous at 20°C (gradient off) and then displayed a gradient between 15°C in the center to 20°C at the edges (gradient on). Similar values were previously used to induce negative thermotaxis (motion towards cold regions) in planarians (Inoue et al., 2014).

Per experiment, two trials were run to compare the behavior of the worms with the gradient turned on and off. Imaging was performed with the same set-up as the behavior assay for 10 minutes at 1fps. Heat maps were generated from the resulting movies by subtracting a background picture without worms and computing the standard deviation projection of the resulting stack in ImageJ (National Institutes of Health). To quantify the amount of thermotaxis, each heatmap was first rescaled to have a mean intensity of 1 to account for possible differences in background lighting. We then computed the ratio of the resulting intensity in the cold region with gradient over the same region without gradient. Thus, ratios greater than one signify increased grouping of the worms towards the center of the dish, indicating successful negative thermotaxis.

Regeneration assay: For each chemical, a regeneration assay was set up with a minimum of n=10 similarly sized planarians at selected nonlethal concentrations. On day 1, planarians were imaged and amputated with an ethanol-sterilized razor blade. Within 3 hours post-amputation, planarians were transferred to 48-well plates, one worm per well, and 200µL of the appropriate concentration of chemical was added to each well. Except during imaging, all worms were stored at 20oC in the dark. Because little regenerative tissue (blastema) is discernible during the first few days, imaging began on day 4. Worms were imaged on days 4-7 on a MZ16FA stereo microscope (Leica), using a SPOT RT3 camera (Model 25.1, Diagnostics Instruments) controlled by SPOT Basic 5.1 software (SPOT Imaging Solutions). The appearance of eyes was manually scored during imaging.

Image analysis of head regeneration dynamics was carried out using a custom semi-automatic MATLAB script in which the area of the blastema was determined using thresholding techniques (Figure S1E-F) based on two images independently analyzed by two people (i.e. average of four data points). Multiple images were analyzed to account for possible variability in analysis. Only worms which remained intact over the course of the experiment, i.e. were not damaged due to manual manipulation or did not undergo asexual reproduction via fission, were used for analysis. If this occurred, a biological replicate was performed and the data from all replicates were combined. To eliminate any bias based on the size of the worm, for each worm, the average blastema area was normalized by the square of the worm's width (Figure S1E), as measured from two images taken on day 1. The rationale behind this normalization is a correlation between blastema size and wound cross-sectional area. Because we do not have access to the worm thickness in our measurements, but thickness scales proportionally to worm width (Figure S1G), we approximated the cross-sectional area using width squared. Widths were

manually measured in ImageJ. The normalized blastema growth rate (1/days, denoted as γ) was determined as the slope of the linear regression of the normalized blastema area for the entire population (including independent experiments) over days 4-7 (Figure S1H). Error bars represent the 99% confidence intervals.

Immunohistochemistry (IHC) experiments: To analyze effects on brain structure and regeneration, IHC was performed on full worms which had incubated in the respective chemical for 8 days or regenerated therein for 15 days. Following each experiment described above, worms were washed three times in 1x planarian water and transferred to a 1.5ml microcentrifuge tube, with approximately eight worms per tube. Planarians were fixed using a modified version of a previously published protocol (Umesono et al., 2013), with all solutions prepared in phosphate buffered saline. In brief, worms were incubated in 2% hydrochloric acid for 5 minutes and 4% paraformaldehyde/5% methanol for 3 hours, both at 4°C with rotation. Worms were then transferred to room temperature and washed twice quickly with 0.3% Triton-X 100, followed by a 15-minute incubation with reduction solution (50mM dithiothreitol, 1% NP-40, and 0.5% SDS) to increase permeabilization. Worms were subsequently washed in 50% methanol for 10 minutes and stored in 100% methanol at -20°C.

Fixed samples were bleached under bright white light for 5-6 hours in 6% hydrogen peroxide, followed by overnight blocking at 4°C in antibody blocking buffer (1% DMSO, 10% fetal bovine serum, 0.1% Tween-20, and 0.3% TritonX-100). To visualize brain structure, worms were subsequently incubated with mouse α-synapsin antibody (Developmental Studies Hybridoma Bank, 3C11, anti-SYNORF1, deposited to the DSHB by Buchner, Erich) diluted 1:500 in antibody blocking buffer, overnight at 4°C. The samples were washed with 0.1% Tween-20 and 0.3% TritonX-100 five times for 20-30 minutes and incubated overnight at 4°C

with Alexa Fluor 488 Goat Anti-Mouse IgG (H+L) secondary antibody (Life Technologies, A-11001), diluted 1:1000 in antibody blocking solution. Worms were washed five times for 20-30 minutes at room temperature with 0.1% Tween-20 and 0.3% TritonX-100 before mounting and imaged on an inverted IX81 spinning disc confocal microscope (Olympus DSU) using an ORCA-ER camera (Hamamatsu Photonics) and Slidebook software (version 5, Intelligent Imaging Innovations, Inc). As worms could be lost or damaged during the course of staining, IHC was performed on at least two biological replicates of treated worms to obtain n greater than or equal to 10.

To analyze the relative size of the brain, we quantified the fraction of the width of the brain over the width of the head (Figure S1I). Quantification was manually performed in ImageJ by analyzing the maximum intensity projections of z-stacks taken with a 10X objective independently by two researchers who did not know which images he or she was analyzing, thus ensuring that experimenter bias could not influence the analysis. Measurement data was compiled and analyzed in Microsoft Excel and MATLAB.

Potency measurement: To summarize our results, we determined the lowest concentrations of each toxicant at which an effect was seen (lowest observed effect level, LOEL), converted to μM, on 17 quantitative read-outs: LC_{50} for full and regenerating worms at four different time points, mean scaled gliding speeds for full and regenerating worms at two different time points, blastema growth rate, eye regeneration, brain structure for full and regenerating worms, and proper thermotaxis. To compare these concentrations over wide ranges, we defined potency as − (concentration in μM).

Statistical Testing: To determine statistical significance in the obtained results for the various assays, we performed a student t-test for pair wise comparison between toxicant

population and controls after verification that the data was normally distributed. All statistical analyses were performed in MATLAB. As this was a pilot study to establish the sensitivity of planarians for toxicological screening, we empirically determined the number of samples used in each assay. Using a post hoc power analysis with Gpower (Erdfelder et al., 1996), we determined that the sample sizes used in unstimulated behavior, regeneration, and brain structure assays were sufficient to detect effects of one standard deviation at the 1% level at a statistical power of 85%, 75% and 62%, respectively.

Results

Overview

The primary objective of this study was to evaluate whether the asexual freshwater planarian Dugesia japonica is a suitable animal model for studying environmental toxicants, particularly developmental neurotoxicants. Therefore, to assess the usefulness of the system, we evaluated the toxicity of ten well-studied substances: dimethyl sulfoxide (DMSO), a classic solvent and known neurotoxicant; pesticides commonly used in agriculture: two organophosphates, chlorpyrifos and dichlorvos, and one pyrethroid, permethrin, because of their relevance for human health and their known toxic mechanisms inhibiting the enzyme acetylcholinesterase and disrupting neuronal sodium channels, respectively (Amitai et al., 1998; Bradberry et al., 2005); the detergents TritonX-100 and sodium dodecyl sulfate (SDS), commonly used in cleaning products and with characterized detrimental effects on fish and other aquatic organisms (Abel, 1974); the most common alcohols, ethanol and methanol, which are well-established to cause developmental neurotoxicity; acrylamide, a widely used industrial chemical also commonly found as a food contaminant (Parzefall, 2008), with known effects as a potential neurotoxicant (LoPachin, 2004); and glucose, expected to be inert to neurodevelopment but potentially affecting other pathways, particularly in metabolism, to establish how effects other than neurotoxicity could be assessed in our system.

We used these compounds to determine (a) how sensitive planarians were to these toxicants when compared to other animal models, and (b) whether a detectable difference existed in the response of adult versus developing planarians, with particular interest in changes in brain structure. To this end, we developed a 5-step semi-automated screening platform that enabled us to first determine the LC_{50} and then the lowest observed effect level (LOEL) for each

compound, using four additional readouts at sublethal concentrations: unstimulated behavior, stimulated behavior, regeneration dynamics, and structural brain defects, as outlined in Figure 2.1.

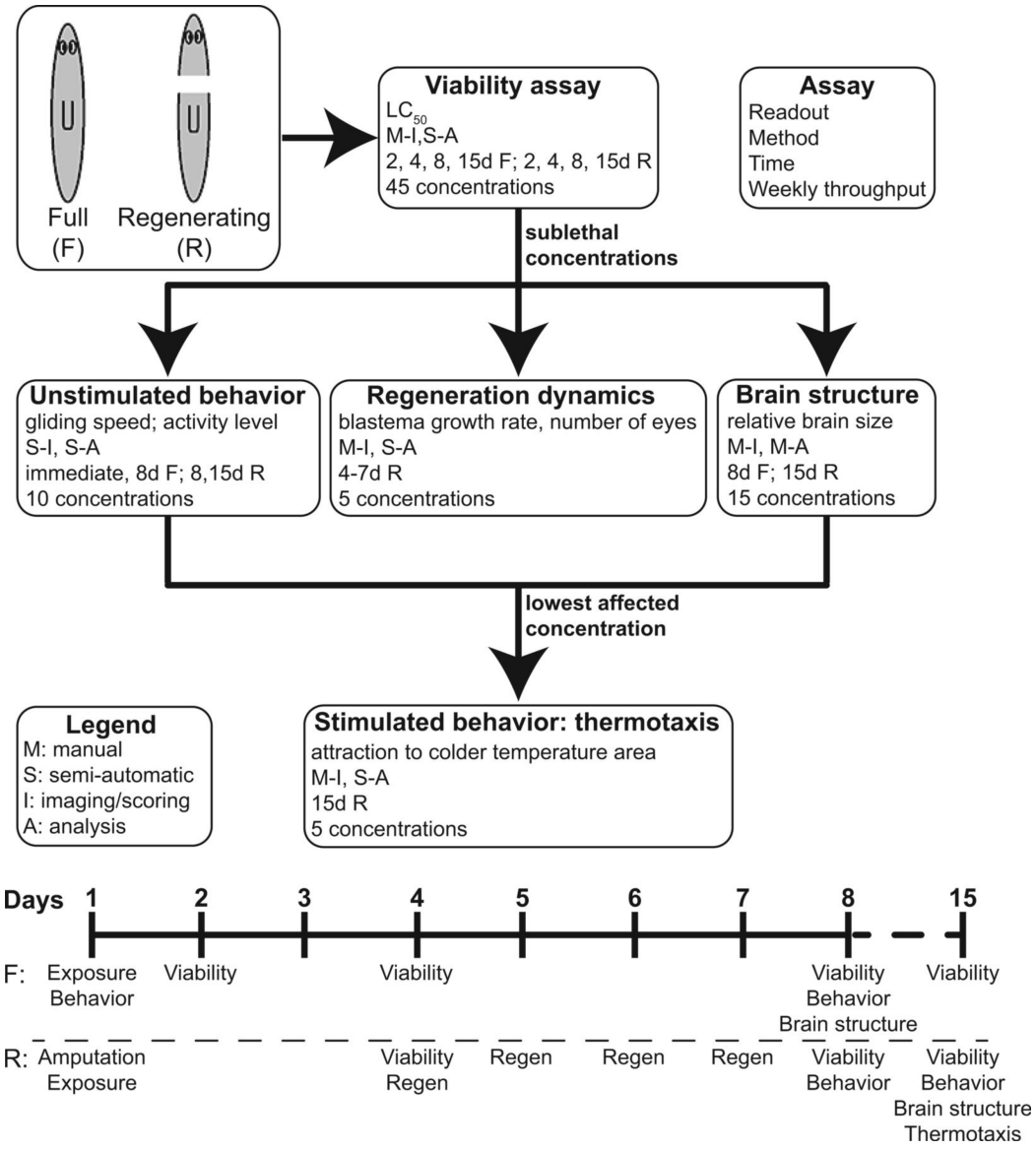

Figure 2.1. Overview of assay. Description of experiments performed with readout, method, times tested, and average weekly throughput listed for each. With the exception of thermotaxis, full and regenerating tail pieces were used for all assays. A timeline is given to describe the screening experimental procedure.

Viability

The first step in our screening platform was to determine the lethal concentration of each compound. Selection of several of the initial broad concentration ranges were guided using previously published reports of lethality and toxicity in planarians (Li, 2008; Pagán et al., 2006; Yuan et al., 2012) and zebrafish (Bichara et al., 2014; DeMicco et al., 2010; Maes et al., 2012; Watson et al., 2014). Since lethality does not solely depend on toxicant concentration but also on the length of exposure, we assessed lethality after 2, 4, 8, and 15 days of exposure (Figure 2.2). Also, we compared the survival of full (adult) and regenerating worms, exposed within 3h post-amputation, over this time scale to assess whether some chemicals were more potent during development. Each chemical was therefore attributed a LC_{50} at four different time points for both full and regenerating worms (n=16 each, from two independent experiments, Table 2.2). As expected, the LC_{50} decreased with the length of exposure. For our other assays, we retained the 15 day LC_{50} as the maximum concentration to be used.

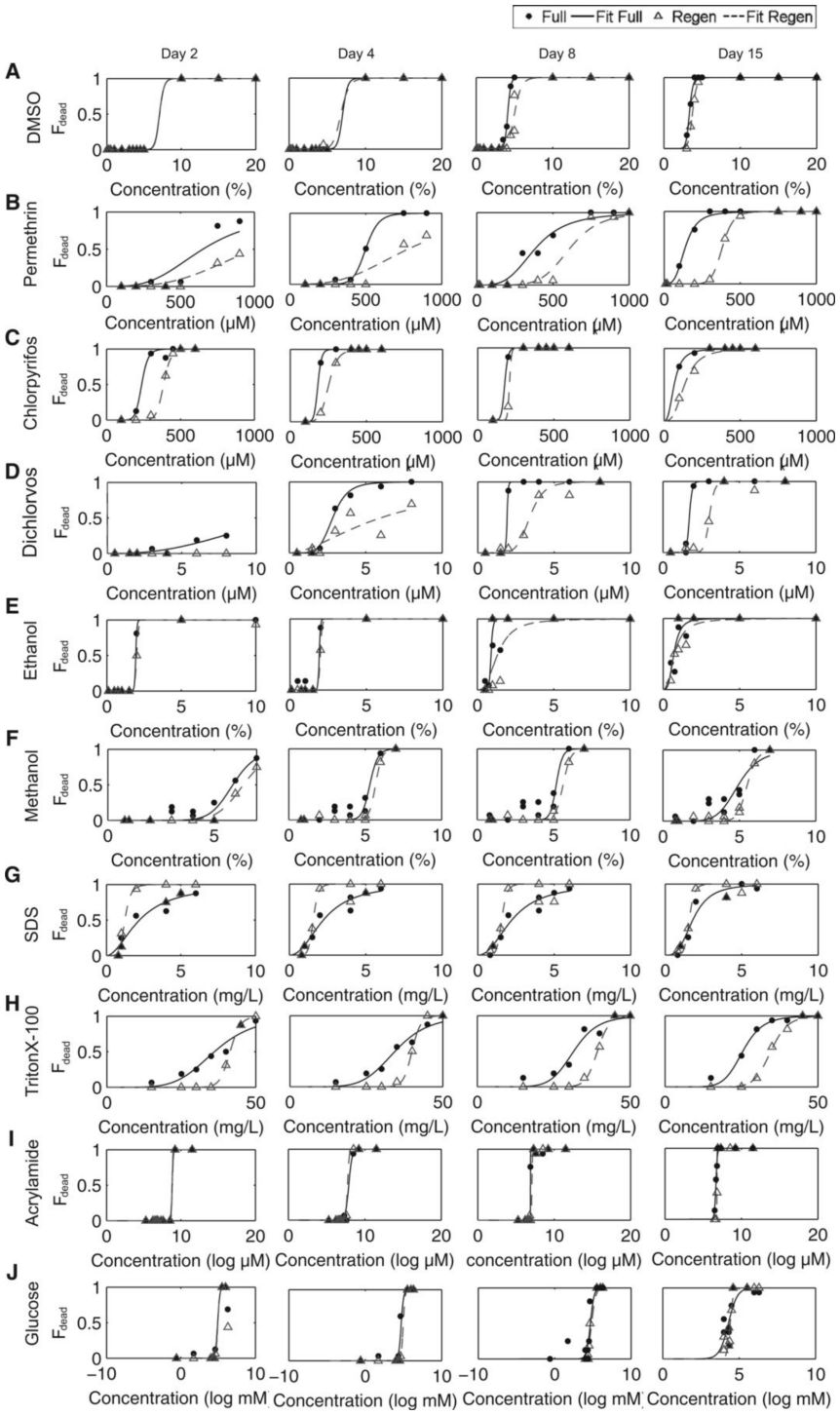

Figure 2.2. Viability of full and regenerating worms. The lethality of each chemical is shown as the fraction of dead worms (Fdead) after 2, 4, 8, or 15 days of exposure to (A) DMSO, (B) permethrin, (C) chlorpyrifos, (D) dichlorvos, (E) ethanol, (F) methanol, (G) SDS, (H) TritonX-100, (I) acrylamide, and (J) glucose for full (black) and regenerating (red) worms. Solid black and red dashed lines show the result of the fit, as described in methods, for full and regenerating worms, respectively.

Table 2.2. LC_{50} values after 2, 4, 8, or 15 days of exposure for full and regenerating worms.

Chemical	Worm condition	Day 2	Day 4	Day 8	Day 15
Acrylamide	Full	6787 µM	2720 µM	991 µM	785 µM
	Regen	6787 µM	1462 µM	1208 µM	904 µM
Chlorpyrifos	Full	238 µM	181 µM	177 µM	67 µM
	Regen	386 µM	252 µM	209 µM	135 µM
Dichlorvos	Full	11.9 µM	2.86 µM	1.92 µM	1.73 µM
	Regen	N/A	6.07 µM	3.40 µM	3.04 µM
DMSO	Full	7.08%	7.03%	4.13%	3.35%
	Regen	7.06%	6.80%	5.03%	3.75%
Ethanol	Full	1.94%	1.92%	0.90%	0.70%
	Regen	2.00%	1.98%	1.34%	0.75%
Glucose	Full	139 mM	110 mM	105 mM	74 mM
	Regen	144 mM	143 mM	125 mM	83 mM
Methanol	Full	5.88%	5.31%	5.18%	4.92%
	Regen	6.38%	5.68%	5.63%	5.51%
Permethrin	Full	653 µM	500 µM	384 µM	139 µM
	Regen	1000 µM	784 µM	609 µM	382 µM
SDS	Full	2.22 mg/l	2.26 mg/l	2.26 mg/l	1.82 mg/l
	Regen	1.24 mg/l	1.57 mg/l	1.57 mg/l	1.57 mg/l
TritonX-100	Full	36 mg/l	34 mg/l	31 mg/l	25 mg/l
	Regen	41 mg/l	40 mg/l	39 mg/l	35 mg/l

LC_{50} was quantified using a modified Hill's equation (see "Materials and Methods" section).
N/A indicates no deaths were observed.

Surprisingly, we found that regenerating worms were slightly more resilient than full worms in the same conditions, with the notable exception of SDS. This effect was most apparent with the pyrethroid permethrin, (Figure 2.2, Table 2.2), where, after 15 days of exposure, the LC_{50} value for regenerating worms (382μM) was found to be almost three times greater than that for full worms (139μM). A possible explanation for this difference in sensitivity may be that regenerating worms are generally more stationary than full worms, potentially reflecting a difference in metabolism.

Notably, we observed a 100-fold difference in LC_{50} values between the two organophosphates, chlorpyrifos and dichlorvos. This difference is potentially due to the differences in the structure and metabolism of these two compounds. Dichlorvos and chlorpyrifos are dimethyl and diethyl organophosphates, respectively; thus, they could potentially have different affinities for planarian acetylcholinesterase. Furthermore, dichlorvos is already in its toxic oxon form whereas chlorpyrifos must be metabolically converted into its oxon by proteins of the cytochrome P450 family to be able to inhibit acetylcholinesterase (Tang et al., 2001), potentially reflecting the observed decreased sensitivity to chlorpyrifos, in comparison to dichlorvos.

Overall, the observed values are comparable to data from zebrafish and C. elegans (see Discussion) demonstrating that planarians are not unusually sensitive or resilient to any of these compounds.

Unstimulated behavior

For the sublethal concentrations determined above, we assayed possible defects in unstimulated planarian behavior induced by the different toxicants through quantification of the

gliding speed and overall activity level of individual worms. Proper gliding requires both a constant production of mucus and coordinated cilia beating. Even recently regenerating worms are capable of gliding, albeit at a reduced speed until 12-13 days of regeneration (Figure S2), showing that gliding does not require a fully functional brain but more likely depends on the function of the ventral nerve cords and proper metabolism.

First, we tested the toxicants' acute general toxicity by measuring the mean gliding speed of full worms immediately after exposure to different sublethal concentrations. Then, to determine the subchronic toxicity of these toxicants, we measured gliding speeds of both full and regenerating worms after 8 days of exposure to distinguish subchronic toxic effects that affected either full or regenerating worms and thus identify possible effects specific to development. Finally, we tested regenerating worms after 15 days of exposure to assess possible delays in the return of normal gliding speeds following amputation.

Acute toxicity was observed as a reduction in gliding speed in 200μM permethrin (Figure 2.3B), 100nM and 500nM dichlorvos (Figure 2.3D), and 0.5mg/L and 1mg/L SDS (Figure 2.3G). As expected, these concentrations also caused decreased gliding speeds on longer time scales in both full and regenerating worms. In addition, acute toxicity was also observed by a decrease in the worms' activity for 1% and 2% DMSO (Figure S3A) and 200μM and 500μM acrylamide (Figure S3B). Here again, similar effects were observed at longer time scales in these conditions.

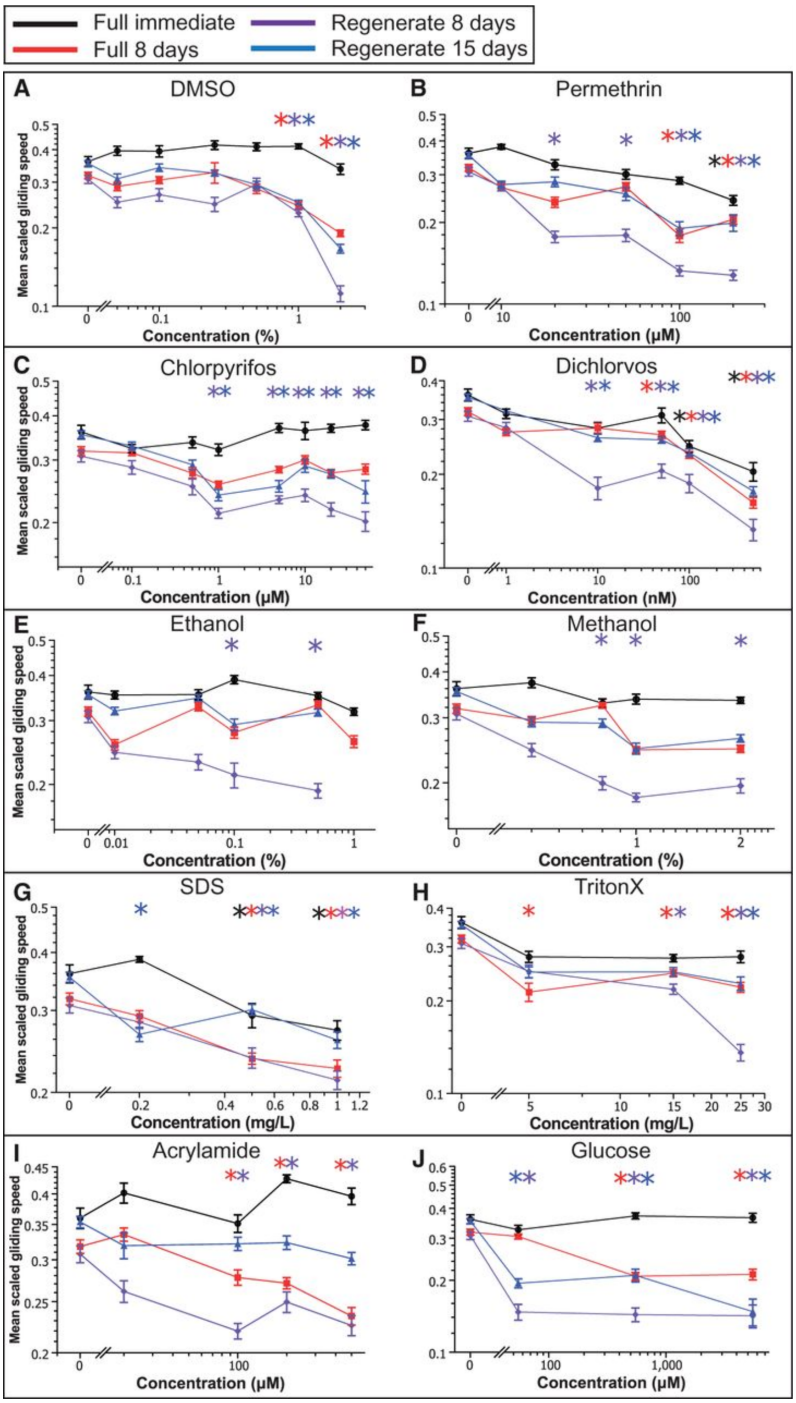

Figure 2.3. Unstimulated behavior of toxicant-exposed full and regenerating worms. Semi-log plot of mean scaled gliding speeds as a function of concentration during exposure to: A, DMSO, B, permethrin, C, chlorpyrifos, D, dichlorvos, E, ethanol, F, methanol, G, SDS, H, TritonX-100, I, acrylamide, and J, glucose. Different graphs correspond to the different time points and situations tested: immediate reaction of full worms, 8 days reaction of full worms and reaction of regenerating worms at both 8 and 15 days. Errors bars are SE of populations of n = 24 worms. Stars indicate statistical relevance at the 1% level for the corresponding time point when compared with control worms.

All tested chemicals displayed subchronic toxicity, demonstrating the sensitivity of our unstimulated behavioral assay. Of the ten tested chemicals, five (DMSO, permethrin, SDS, TritonX-100, and glucose) showed subchronic toxicity in all conditions with slight differences in threshold concentrations between regenerating and full worms. The fact that subchronic exposure to glucose resulted in perturbed behavior was expected given its central role in metabolism, which directly affects unstimulated behavior. More specifically, of these five chemicals, all except TritonX-100, displayed lower threshold concentrations in regenerating worms, indicating possible increased sensitivity of developing planarians to these chemicals. However, the other five toxicants had more surprising toxicity profiles.

The alcohols, methanol and ethanol, were peculiar in the sense that they only affected 8 days regenerating worms (above 0.8% and 0.1%, respectively) but neither full nor 15 days regenerating worms (Figure 2.3E-F), suggesting that these concentrations induced a slight delay in the retrieval of locomotion function during regeneration but did not impair these functions altogether.

The organophosphates, chlorpyrifos and dichlorvos, were particularly interesting since regenerating worms showed a higher sensitivity to these class of toxicants when compared to full worms (either immediately or after 8 days of exposure). Chlorpyrifos was the most striking with concentrations as low as 1μM inducing reduced gliding speeds in both 8 days and 15 days regenerating worms whereas none of the tested concentrations showed any effect on full worms (Figure 2.3C). In addition, qualitative differences in the worm's trajectories were visible in chlorpyrifos with an increased frequency of sharp turns and head wiggles (Figure S3C-D), similar to reports of a zigzag swimming pattern seen in zebrafish larvae exposed to chlorpyrifos (Watson et al., 2014). Similarly, regenerating worms were more sensitive to dichlorvos than full

worms (Figure 2.3D). These results support the hypothesis that organophosphates might have developmental specific neurotoxic effects (Bjørling-Poulsen et al., 2008; Richendrfer et al., 2012) whose mechanisms remain to be understood.

Finally, acrylamide only showed subchronic toxicity on 8 days full and regenerating worms at concentrations higher than 100μM (Figure 2.3I). However, this effect was coupled to a clear reduction of activity levels (seen as the increased fraction of time spent resting, see Figure S3B) in full and regenerating worms, at both 8 and 15 days. These results suggest a more subtle effect of acrylamide on unstimulated behavior with potential effects on both the type of behavior adopted by the worms and their ability to perform gliding normally.

Altogether, these results show the ability of our semi-automated setup to reveal subtle effects on passive behavior due to toxicant exposure. We were able to distinguish acute and subchronic toxicity as well as reveal defects specific to developing brains. This emphasizes the strength of the opportunity offered by planarians to study, in parallel and at medium throughput, both adult and regenerating organisms.

Regeneration/development dynamics

Since we are using asexual D. japonica planarians, regeneration of a new brain after amputation is comparable to the typical development of a new planarian brain after "birth", which is the generation of a tail piece during binary fission (Sakurai et al., 2012). Thus, by assaying brain regeneration, we are, in a way, simultaneously assaying brain development. To test whether any of the chemicals had adverse effects on regeneration dynamics and therefore development, amputated planarians were exposed to our pre-determined sublethal range of concentrations for each chemical for 7 days, during which regeneration dynamics and eye

reappearance were quantified as outlined in Material and Methods (see Figure S1J-L for example images). Since proper regeneration requires the coordination of many different processes, including stem cell proliferation, differentiation, and re-establishment of polarity (Reddien, 2013; Umesono et al., 2013), possible toxic effects on this process are likely due to mechanisms of general developmental toxicity. Moreover, while equally regulated by the same processes as general regeneration, eye regeneration, is coordinated by specific neuronal populations (Dong et al., 2012; Mannini et al., 2004) and is therefore a more sensitive endpoint to assay possible specific neurotoxic effects. Therefore, this combined quantitative analysis of regeneration allowed us to simultaneously assess general physiological developmental toxicity as well as specific neuronal toxicity.

Surprisingly, most of the tested chemicals did not have a significant effect on either the normalized blastema growth rate (γ) or the number of eyes detected at day 7 (Figure 2.4). Of the tested chemicals and concentrations, only 1% DMSO and 15mg/L TritonX-100 (Figure SIK) caused a significant delay in blastema growth. Similarly, at these same concentrations, more worms were found to have delays in eye regeneration, as a large number of worms had only one or no eyes at day 7, whereas the majority of controls had regenerated both eyes (Figure 2.4C and L).

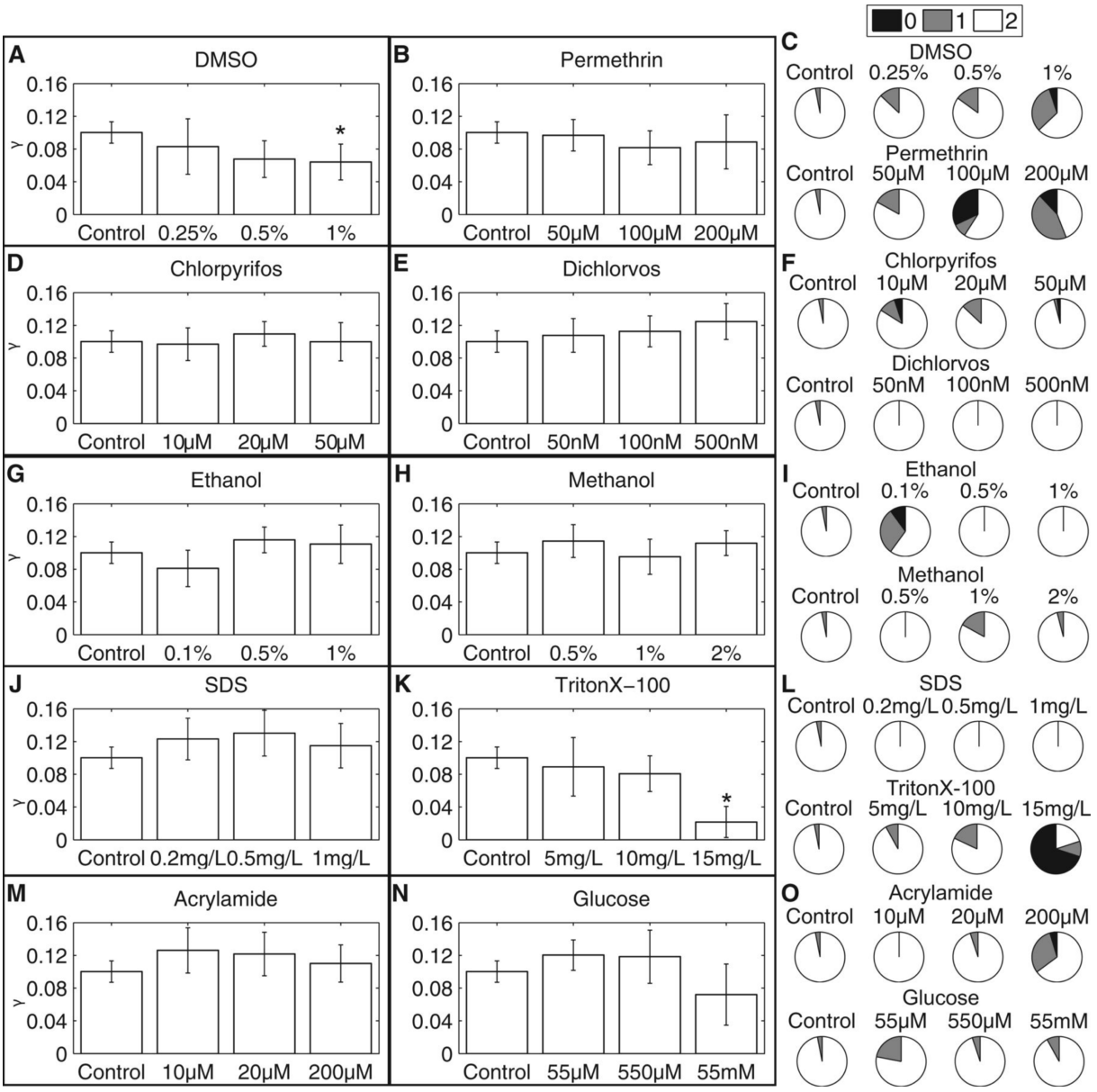

Figure 2.4. Regeneration is generally unaffected by toxicant exposure. Effects of the various chemicals on regeneration were quantified by the population blastema growth rate over days 4–7, normalized by the worm width squared, (γ), and the percent of worms with 0, 1, or 2 eyes at day 7 for: A–C, DMSO (n = 15, 20, and 19) and permethrin (n = 12, 22, and 9), D–F, chlorpyrifos (n = 19, 31, 34) and dichlorvos (n = 12, 20, and 11), G and H, ethanol (n = 20, 24, and 11) and methanol (n = 11, 12, and 24), J and K, SDS (n = 12, 12, and 10) and TritonX-100 (n = 12, 11, and 10), and M and N, acrylamide (n = 20, 20, and 10) and glucose (n = 18, 20, and 12) compared with controls (n = 58). Error bars represent the 99% confidence intervals of the fit. *Denotes the confidence intervals do not overlap with those of controls.

Interestingly, although no significant effect on blastema growth was found, worms regenerated in 100μM and 200μM permethrin and 200μM acrylamide showed a delay in eye regeneration (Figure 2.4C, O, and S1L), suggesting that the effects of permethrin and acrylamide may be more specifically neurotoxic rather than generally toxic. This is consistent with the known effects of pyrethroids on neuronal voltage-gated sodium channels (Bradberry et al., 2005) and acrylamide on axonal swelling and demyelination (LoPachin, 2004; Parng et al., 2007).

In general, we found that the majority of the tested toxicants were not toxic to the overall physiology of the regenerating planarian. This suggests that, at the concentrations tested, any adverse effects seen in the toxicant-treated regenerating worms may be due to more targeted effects on specific pathways, rather than an effect of general toxicity.

Brain structure

A powerful tool of alternative model organisms, such as zebrafish, nematodes, and planarians, is the ability to probe toxicity at different levels, from the organismal level down to the cellular and molecular level. To evaluate whether subchronic exposure to sublethal concentrations of the tested chemicals could lead to obvious morphological changes in the planarian brain, indicating possible brain defects resulting from toxicant exposure, we visualized the nervous system by immunohistochemistry with a pan-neuronal marker, α-synapsin. To account for differences in worm size, the relative brain size was calculated as the ratio of the width of the brain to the width of the head at the same location (Figure S1I). Importantly, through this quantitative analysis, we were able to detect neurotoxicity manifested by large scale defects in the gross anatomy of the brain; however, more subtle neurotoxicity at the cellular level could be missed including defects in specific neurodevelopmental processes, such as neurite

outgrowth or synaptogenesis.

We compared the relative brain size of full and regenerating worms exposed to different concentrations for 8 and 15 days, respectively (Figure 2.5). These time-scales were chosen as behavioral defects were detectable after 8 days for both full and regenerating animals (Figure 2.3). However, for regenerating animals, toxicant exposure could potentially slow brain reformation. To specifically analyze toxic effects on brain morphology, rather than developmental delays, regenerating worms were assayed after 15 days of exposure to allow for complete nervous system regeneration. Full worms were tested to allow for comparison with regenerating worms to determine whether the toxicants were specific to either the developing or mature brain or were general to both.

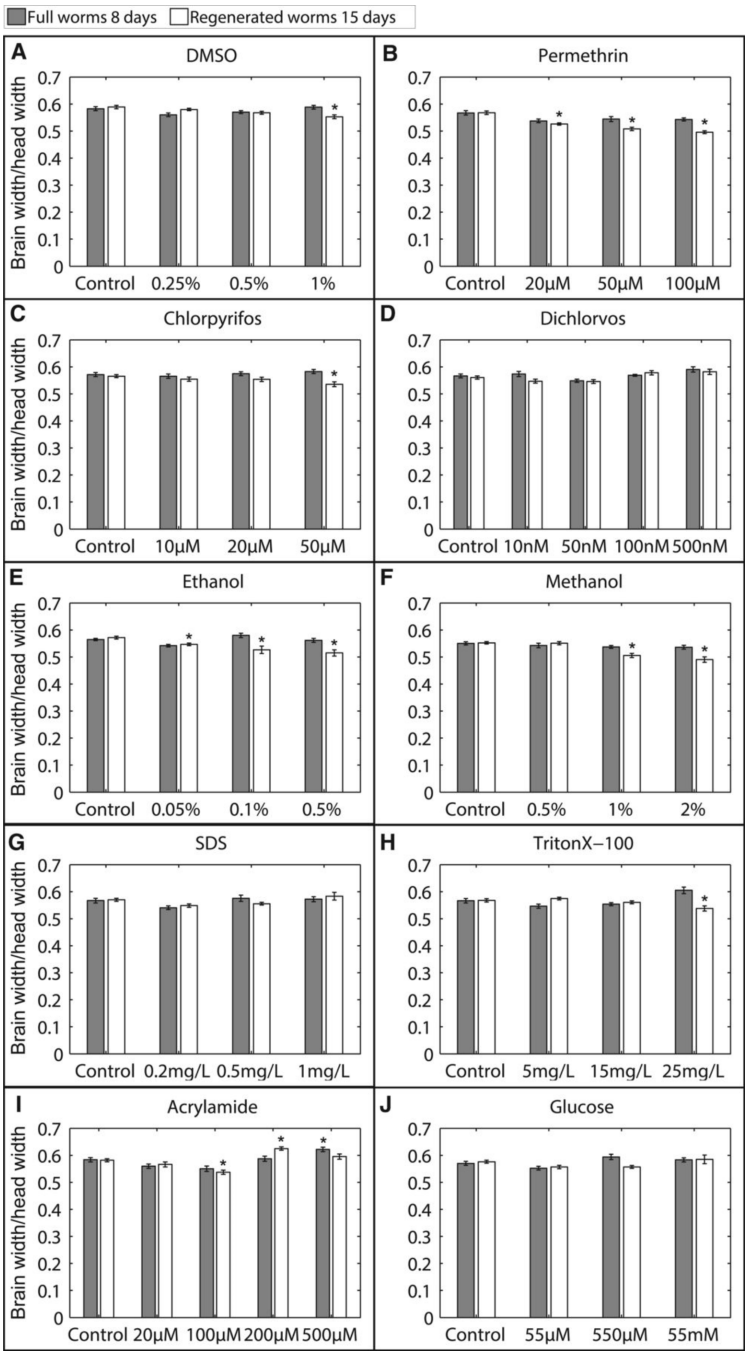

Figure 2.5. Effects on brain morphology. Quantification of relative brain size as brain width/head width comparing controls (n = 20 full and 30 regenerating worms) to animals exposed to: A, DMSO (n = 11, 14, 13; 13, 15, 10), B, permethrin (n = 13, 13, 13; 15, 11, 13), C, chlorpyrifos (n = 10, 11, 16; 14, 21, 11), D, dichlorvos (n = 12, 13, 16, 12; 17, 16, 10, 11), E, ethanol (n = 19, 13, 10; 16, 19, 11), F, methanol (n = 12, 22, 20; 11, 11, 13), G, SDS (n = 12, 12, 11; 17, 15, 11), H, TritonX-100 (n = 14, 19, 13; 16, 10, 15), I, acrylamide (n = 15, 14, 19, 15, 12; 19, 16, 12, 14, 13), and J, glucose (n = 13, 17, 13; 19, 13, 13). n listed as (full; regenerated worm) in increasing concentration order. Error bars denote SE and * denotes $p < .01$ when compared with controls of the same worm type.

Generally, after toxicant exposure, brain morphology was more sensitively affected in regenerating worms than in full worms treated with the same concentrations. Development-specific defects in brain size, wherein regenerating but not full worms were affected, were detected after exposure to DMSO, permethrin, chlorpyrifos, ethanol, methanol, and TritonX-100 (Figure 2.5).

This increased sensitivity displayed by regenerating worms was especially evident in worms exposed to permethrin, ethanol, and methanol, wherein a significant decrease in brain size was detected at multiple tested concentrations, although, even at the highest tested sublethal concentrations, no changes in the full worm brain morphology were found. Notably, although no quantitative differences in brain size were detected for regenerated worms treated with dichlorvos, qualitative differences in brain density were observed (Figure S4), indicating possible neurotoxicity which would require more in-depth analysis at the molecular or cellular level. Overall, the chemicals we tested were more potent on developing brains than on adult ones underlying the need for specific guidelines controlling exposure of infants and pregnant women to various toxicants.

Compared to exposure to the other chemicals, which resulted in classical dose-dependent changes in regenerated brain size, exposure to acrylamide was special with a seemingly biphasic effect on brain size. In fact, we found that exposure to lower concentrations of acrylamide (notably,100μM) led to a significant decrease in regenerated brain size; however, exposure to high concentrations (200μM) resulted in an increase in regenerated brain size compared to non-treated controls (Figure 2.5I). Upon inspection of the respective images associated with these brains, this effect was clearly visible as developing brains incubated in 200μM acrylamide seemed to have a swollen and wider distribution of neurons, compared to control and lower

concentrations of acrylamide (Figure S4). This effect is consistent with the previously described ability of acrylamide to cause axonal swelling (Parng et al., 2007). Furthermore, of all the tested concentrations in the various chemicals, only 500μM acrylamide caused significant morphological changes in the adult brain. Similar to the effects with high concentrations of acrylamide on regenerating brains, this concentration induced an increase in brain size compared to controls, suggesting similar mechanisms of toxicity are occurring in the developing and adult brain, although with different sensitivities.

Full or regenerating worms exposed to sublethal concentrations of SDS did not display significant changes in brain morphology (Figure 2.5G); however, more subtle effects on brain structure or function (see below) could be present which we would be unable to discern by this large-scale morphological approach. This was similarly seen for the non-toxic, neutral chemical, glucose (Figure 2.5J), wherein we did not expect to find any structural changes in the brain.

Overall, quantitative comparison of relative brain sizes in regenerating and full worms allowed us to detect large-scale developmental-specific effects of neurotoxicity as exposure at the same concentrations specifically affected the brain size of regenerating animals.

Stimulated behavior: thermotaxis

Since the neuronal processes involved in unstimulated behavior are likely limited, evidenced by the ability of regenerating worms without a fully reformed brain to glide (Figure S2), we analyzed the ability of worms exposed to the various toxicants to perform temperature sensing as a more subtle readout of neuronal function. It has been previously shown (Inoue et al., 2014) that wild-type planarians exhibit a strong preference for colder temperatures; therefore, we tested for proper brain function using the worms' negative thermotaxis, i.e. their ability to move

towards regions of lower temperature. The neuronal mechanisms underlying planarian thermotaxis involve temperature sensing by receptors of the transient receptor potential family, signal processing by serotoninergic neurons in the brain, and behavioral output mediated by cholinergic motor neurons (Inoue et al., 2014). The ability of a worm to perform negative thermotaxis is thus a good readout of the proper function of these specific sensory and processing neurons. We tested thermotaxis on worms that were allowed to regenerate for 15 days in the presence of the different toxicants. Because these tests were conducted manually, as described in Material and Methods, we only tested one concentration per chemical using either the lowest concentration found to induce defects in brain morphology or found to induce behavioral abnormalities for 15 days regenerating animals.

Through quantification of the worms' response and visual inspection of the density heat maps (Figure 2.6A-B, Figure S5), we found that thermotactic ability was entirely suppressed after exposure to 0.5% ethanol, 50μM chlorpyrifos, and 25mg/L TritonX-100 (dark grey bars in Figure 2.6C). In addition, we found that this behavior was impaired but not entirely suppressed after exposure to 55μM glucose, 2% methanol, and 1mg/L SDS (light grey bars in Figure 2.6C).

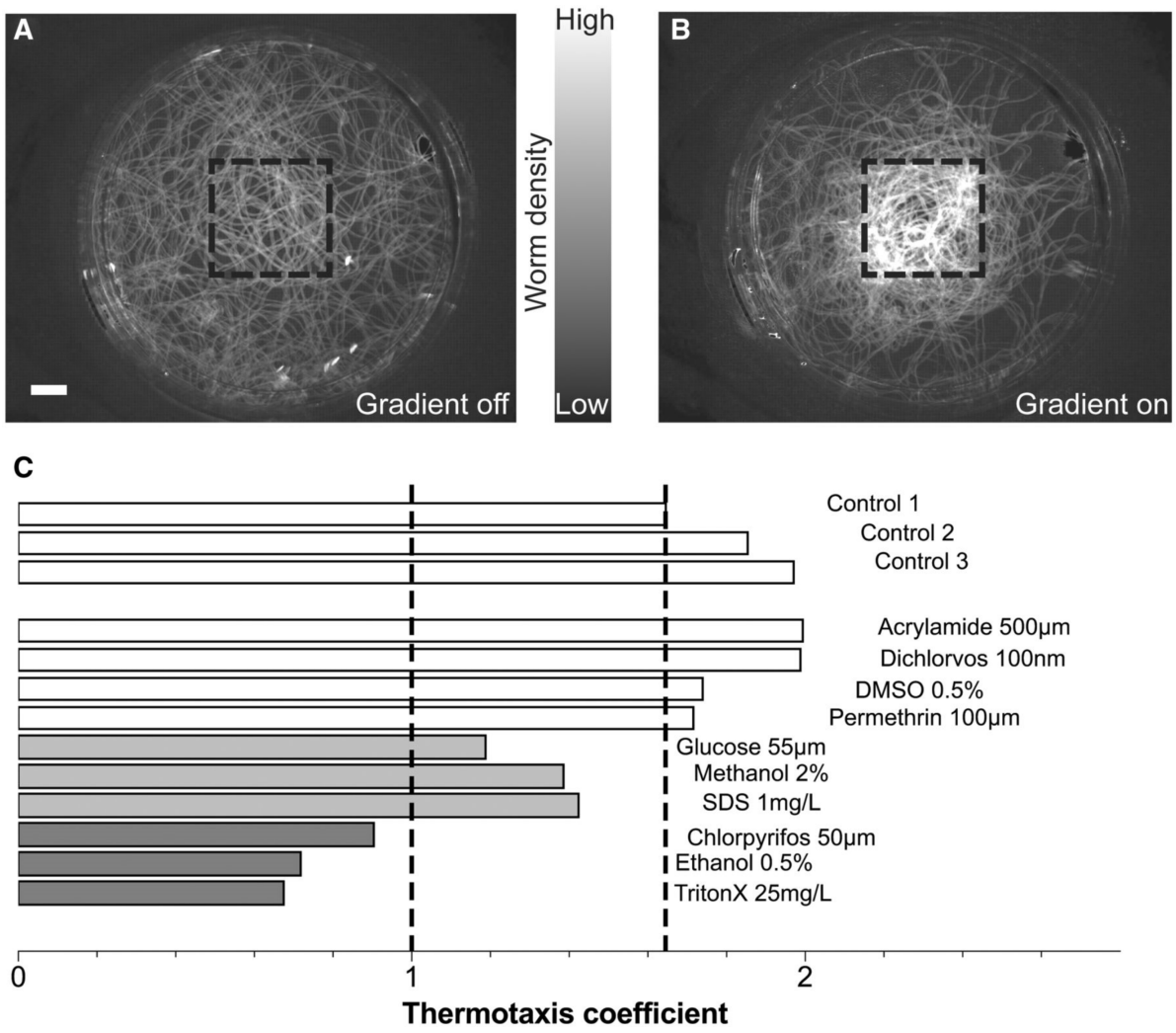

Figure 2.6. Temperature sensing assay. A, Wild-type worms (n = 20) density heatmap over a 10-min course in the absence or B, presence of a thermal gradient. Black dotted line shows the area of the cold spot in the center of the dish and gray levels indicate higher worm density in that region in presence of the gradient. Scale bar: 1 cm. C, Thermotaxis coefficient for worm populations (n = 20 for each) exposed to different toxicants. The black dotted lines indicate the level of absence of any reaction (thermotaxis coefficient of 1) and the lowest measurement of 3 control populations. The different conditions are further classified based on these 2 cutoffs as normal thermotaxis (white bars), impaired thermotaxis (light gray bars) and no thermotaxis (dark gray bars).

Of these six toxicants, four (ethanol, methanol, chlorpyrifos, and TritonX-100) were already shown to induce large scale defects in brain morphology (Figure 2.5), likely explaining this impaired behavior. On the other hand, the structural defects induced by DMSO, permethrin, and acrylamide did not impair thermotaxis and, therefore, are likely targeted at different neuronal populations, not involved in this type of behavior. Finally, at the tested concentrations, neither glucose nor SDS induced visible changes in brain morphology but still impaired thermotaxis. This effect of glucose could potentially be explained by its role in the insulin pathway which has been shown to play a role in thermotaxis and memory in C. elegans (Li et al., 2013). In addition, both glucose and SDS were found to have effects on locomotion (Figure 2.3) which could also alter the worms' thermotactic response which, ultimately, requires proper motility.

Overall, these results show how planarians can be used in large scale, population experiments which, in concordance with our other assays, reveal subtler effects on neuronal functions. In the future, similar tests could be conducted using the worm's photo- or chemo-tactic responses which require different neuronal subpopulations to further refine the neurotoxicity profiles of various toxicants.

Discussion

As shown in Figure 2.7, all of the tested toxicants displayed some form of toxicity demonstrated through either unstimulated or stimulated behavior, regeneration dynamics, or brain structure indicating that planarians are an appropriately sensitive animal model for toxicology studies. Importantly, the tested toxicants displayed differential toxicity with different levels of effect on the various endpoints, suggesting these endpoints are specific to various types of toxicity, ranging from general physiological toxicity (regeneration dynamics) to toxicity towards specific neuronal subpopulations (thermotaxis).

Moreover, comparison with other toxicology model organisms, such as zebrafish and nematodes, shows that planarians generally displayed comparable sensitivity to the tested toxicants, with LC_{50} and LOEL values on the same order of magnitude (Tables 2.3-2.4). However, species-specific differences in sensitivity do exist, most strikingly in the case of permethrin. Although, in terms of lethality, planarians were 1000 fold less sensitive than zebrafish to permethrin, it has been shown that fish are particularly sensitive to pyrethroid exposure, with a 1000-fold higher sensitivity than mammals (Bradbury and Coats, 1989). This emphasizes the need for a comparative analysis of toxicology across diverse model organisms to better represent possible effects on humans and to find the appropriate threshold concentrations.

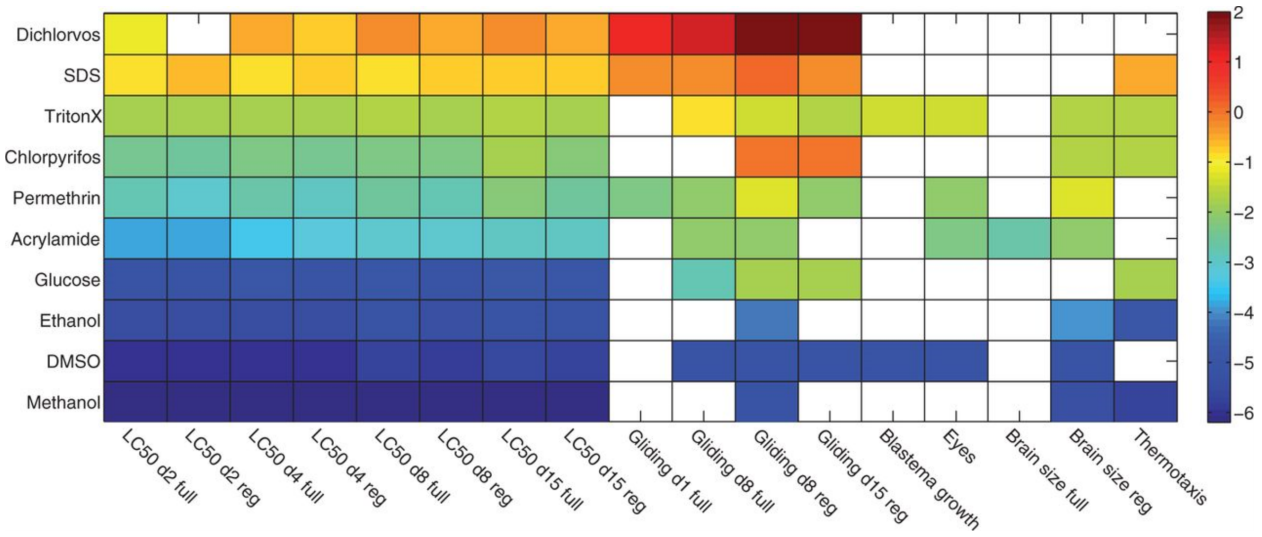

Figure 2.7. Effect and potency of all toxicants on 10 quantitative endpoints: LC_{50} for full and regenerating worms at 4 different time points, mean scaled gliding speeds for full and regenerating worms, blastema growth rate, eye regeneration, brain structure for full and regenerating worms and, finally, proper thermotaxis. The colorbar represents potency defined as $-\log_{10}$ (LOEL in µM) (see "Materials and Methods" section), whereas white squares are used when no effects were detected.

Table 2.3. Comparison of LC_{50} values for planarians with zebrafish and nematodes.

Chemical	Full planarians	Regenerating planarians	Zebrafish	Nematodes	References
DMSO	4.13%	5.03%	1.8–2.5%		Bichara *et al.*(2014) and Maes *et al.* (2012)
Permethrin	384 µM	609 µM	800 nM		DeMicco *et al.*(2010)
Chlorpyrifos	177 µM	209 µM	1 µM	2.76 µM	Roh and Choi (2008) and Watson *et al.* (2014)
Dichlorvos	1.92 µM	3.40 µM	17 µM	39 µM	Rajini *et al.*(2008) and Watson *et al.* (2014)
Ethanol	0.9%	1.34%	1.2%	5%	Bichara *et al.*(2014) and Yu *et al.* (2011)
Methanol	5.18%	5.63%			
SDS	2.26 mg/l	1.57 mg/l	16.1 mg/l		Bichara *et al.*(2014)
TritonX-100	31 mg/l	39 mg/l			
Acrylamide	785 µM	904 µM	~6.25 mM	3.4 mM	Fei *et al.* (2010)and Li *et al.*(2015)
Glucose	105 mM	125 mM			

LC_{50} values of 8 day full and regenerating planarians, from Table 2.2, are compared with values found in zebrafish larvae and nematodes. When necessary, concentrations were converted for better comparison.

Table 2.4. Comparison of LOEL Values of Tested Chemicals in Planarians with in Zebrafish and Nematodes

Chemical	Planarians	Zebrafish	Nematodes	References
DMSO	1%	0.01–2%	1%	Chen *et al.* (2011), Maes *et al.*(2012), Selderslaghs *et al.* (2009), and Sprando *et al.* (2009)
Permethrin	20 μM	130 nM		DeMicco *et al.* (2010)
Chlorpyrifos	1 μM	0.01–0.1 μM	0.029 μM	Richendrfer *et al.* (2012), Roh and Choi (2008), and Watson *et al.* (2014)
Dichlorvos	10 nM	0.1 μM	1.2 nM	Rajini *et al.* (2008) and Watson *et al.* (2014)
Ethanol	0.05%	0.01–1%	0.1%	Chen *et al.* (2011), Chromcova *et al.* (2012), Dhawan *et al.*(1999), and Maes *et al.* (2012)
Methanol	0.8%	1%	2%	Chromcova *et al.* (2012), Katiki *et al.* (2011), and Maes *et al.*(2012)
SDS	0.2 mg/l	6.4 nM (~1.8 mg/l)		Truong *et al.* (2014)
TritonX-100	5 mg/l			
Acrylamide	100 μM		141 μM	Li *et al.* (2015)
Glucose	55 μM	> 55 mM	250 mM	Mondoux *et al.* (2011) and Selderslaghs *et al.* (2009)

LOEL determined as the lowest concentration which elicited a statistically significant effect compared with controls. When necessary, concentrations were converted for better comparison.

Species-related sensitivities may reflect differences in toxicokinetics in these different animal models, including toxicant uptake and metabolism. In planarians, the toxicants reach their target tissue by absorption through the skin and diffusion; however, future studies are needed to precisely determine the amount of chemicals taken up and processed by the animal.

In summary, we have shown that the freshwater planarian D. japonica is a suitable alternative animal model for developmental neurotoxicology. While planarians do not have the morphological richness of zebrafish larvae (Truong et al., 2014), thus limiting morphological readouts, they have other unique features that make them a relevant model system: (1) the ability to test adult and developing animals, in parallel, allows us unprecedented insight into development specific effects of toxicants whose molecular and cellular basis remains to be explored in mechanistic studies and (2) because planarians are invertebrates but still possess significant neuronal complexity and homology to the human brain (Buttarelli et al., 2008), they allow us to conduct MTS studies to assess the toxicity of new compounds in a relevant context without the ethical dilemma that comes from working with vertebrate animals. To achieve the necessary throughput and specificity, our current assay clearly needs to be modified in two ways: (1) the different manual components must be integrated into an automated plate handling and scoring platform, and (2) additional readouts, e.g. phototaxis, chemotaxis, etc., must be added to the screen and quantitatively evaluated. Now that we have established the suitability of freshwater planarians as an animal model for developmental neurotoxicology, we plan on starting this second phase of system development.

Acknowledgements

Chapter 2, in full, is a reformatted reprint of the material as it appears in Toxicological Sciences 2015 (Hagstrom, Danielle; Cochet-Escartin, Olivier; Zhang, Siqi; Khuu, Cindy; and Collins, Eva-Maria S. "Freshwater planarians as an alternative animal model for neurotoxicology", Toxicological Sciences, vol. 147, 2015). The version of record is available online at: https://academic.oup.com/toxsci/article/147/1/270/1642148. Use of this manuscript in the dissertation herein is covered by the rights permitted to the authors by Oxford Journals. The dissertation author was the co-author of this paper. Danielle Hagstrom, Olivier Cochet-Escartin, Siqi Zhang and Eva-Maria S. Collins designed and performed the experiments, analyzed and interpreted the data. Cindy Khuu helped with experiments and data analysis. The authors thank the following undergraduate students (Mary B. Tamme, M. Phuong Truong, Innkyu Moon, Jannet Cardin, David Duplantier, Yingtian He) and high school student (Milena Chakraverti-Wuerthwein) who helped with feeding, cleaning, and imaging worms, running some assays, and analyzing imaging data, and Angel Leu for help with IHC. The anti- SYNORF1 antibody developed by Erich Buchner was obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242.Danielle Hagstrom and Olivier Cochet-Escartin were the primary authors of this material.

References

Abel, P. D. (1974). Toxicity of synthetic detergents to fish and aquatic invertebrates. J. Fish Biol. 6, 279–298.

Amitai, G., Moorad, D., Adani, R. and Doctor, B. P. (1998). Inhibition of Acetylcholinesterase and Butyrylcholinesterase by Chlorpyrifos-oxon. Biochem. Pharmacol. 56, 293–299.

Bichara, D., Calcaterra, N. B., Arranz, S., Armas, P. and Simonetta, S. H. (2014). Set-up of an infrared fast behavioral assay using zebrafish (Danio rerio) larvae, and its application in compound biotoxicity screening. J. Appl. Toxicol. 34, 214–9.

Bjørling-Poulsen, M., Andersen, H. R. and Grandjean, P. (2008). Potential developmental neurotoxicity of pesticides used in Europe. Environ. Health 7, 50.

Boyd, W., Smith, M. and Freedman, J. (2012). Caenorhabditis elegans as a Model in Developmental Toxicology. In Developmental Toxicology (ed. Harris, C. and Hansen, J. M.), pp. 15–24. Humana Press.

Bradberry, S. M., Cage, S. A., Proudfoot, A. T. and Vale, J. A. (2005). Poisoning due to pyrethroids. Toxicol. Rev. 24, 93–106.

Bradbury, S. P. and Coats, J. R. (1989). Comparative toxicology of the pyrethroid insecticides. Rev. Environ. Contam. Toxicol. 108, 133–177.

Buttarelli, F. R., Pellicano, C. and Pontieri, F. E. (2008). Neuropharmacology and behavior in planarians: Translations to mammals. Comp. Biochem. Physiol. - C Toxicol. Pharmacol. 147, 399–408.

Cebrià, F. (2007). Regenerating the central nervous system: how easy for planarians! Dev. Genes Evol. 217, 733–48.

Cebrià, F. and Newmark, P. A. (2005). Planarian homologs of netrin and netrin receptor are required for proper regeneration of the central nervous system and the maintenance of nervous system architecture. Development 132, 3691–703.

Cebrià, F., Nakazawa, M., Mineta, K., Ikeo, K., Gojobori, T. and Agata, K. (2002). Dissecting planarian central nervous system regeneration by the expression of neural-specific genes. Dev. Growth Differ. 44, 135–46.

Chen, T.-H., Wang, Y.-H. and Wu, Y.-H. (2011). Developmental exposures to ethanol or dimethylsulfoxide at low concentrations alter locomotor activity in larval zebrafish: implications for behavioral toxicity bioassays. Aquat. Toxicol. 102, 162–6.

Chromcova, L., Stepanova, S., Plhalova, L., Praskova, E. and Svobodova, Z. (2012). Effect of four selected carrier solvents on embryonal stages of Danio rerio. Neuro Endocrinol. Lett. 33 Suppl 3, 60–5.

Collins, F. S., Gray, G. M. and Bucher, J. R. (2008). Toxicology. Transforming environmental health protection. Science 319, 906–7.

DeMicco, A., Cooper, K. R., Richardson, J. R. and White, L. A. (2010). Developmental neurotoxicity of pyrethroid insecticides in zebrafish embryos. Toxicol. Sci. 113, 177–186.

Dhawan, R., Dusenbery, D. B. and Williams, P. L. (1999). Comparison of lethality, reproduction, and behavior as toxicological endpoints in the nematode Caenorhabditis elegans. J. Toxicol. Environ. Health. A 58, 451–62.

Dong, Z., Yuwen, Y., Wang, Q., Chen, G. and Liu, D. (2012). Eight genes expression patterns during visual system regeneration in Dugesia japonica. Gene Expr. Patterns 12, 1–6.

Dunkel, J., Talbot, J. and Schötz, E.-M. (2011). Memory and obesity affect the population dynamics of asexual freshwater planarians. Phys. Biol. 8, 026003.

Erdfelder, E., Faul, F. and Buchner, A. (1996). GPOWER: A general power analysis program. Behav. Res. Methods, Instruments, Comput. 28, 1–11.

Fei, X.-C., Song, C. and Gao, H.-W. (2010). Transmembrane transports of acrylamide and bisphenol A and effects on development of zebrafish (Danio rerio). J. Hazard. Mater. 184, 81–8.

Inoue, T., Yamashita, T. and Agata, K. (2014). Thermosensory Signaling by TRPM Is Processed by Brain Serotonergic Neurons to Produce Planarian Thermotaxis. J. Neurosci. 34, 15701– 15714.

Judson, R. S., Houck, K. A., Kavlock, R. J., Knudsen, T. B., Martin, M. T., Mortensen, H. M., Reif, D. M., Rotroff, D. M., Shah, I., Richard, A. M. and Dix, D.J. (2010). In vitro screening of environmental chemicals for targeted testing prioritization: the ToxCast project. Environ. Health Perspect. 118, 485–92.

Katiki, L. M., Ferreira, J. F. S., Zajac, A. M., Masler, C., Lindsay, D. S., Chagas, A. C. S. and Amarante, A. F. T. (2011). Caenorhabditis elegans as a model to screen plant extracts and compounds as natural anthelmintics for veterinary use. Vet. Parasitol. 182, 264–8.

Knight, A. W., Little, S., Houck, K., Dix, D., Judson, R., Richard, A., McCarroll, N., Akerman, G., Yang, C., Birrell, L. and Walmsley, R.M. (2009). Evaluation of high-throughput genotoxicity assays used in profiling the US EPA ToxCast chemicals. Regul. Toxicol. Pharmacol. 55, 188–99.

Li, M.-H. (2008). Effects of nonionic and ionic surfactants on survival, oxidative stress, and cholinesterase activity of planarian. Chemosphere 70, 1796–803.

Li, Y., Zhao, Y., Huang, X., Lin, X., Guo, Y., Wang, D., Li, C. and Wang, D. (2013). Serotonin control of thermotaxis memory behavior in nematode Caenorhabditis elegans. PLoS One 8, e77779.

Li, J., Li, D., Yang, Y., Xu, T., Li, P. and He, D. (2015). Acrylamide induces locomotor defects and degeneration of dopamine neurons in Caenorhabditis elegans. J. Appl. Toxicol.

LoPachin, R. M. (2004). The changing view of acrylamide neurotoxicity. Neurotoxicology 25, 617–30.

Lowe, J. R., Mahool, T. D. and Staehle, M. M. (2015). Ethanol exposure induces a delay in the reacquisition of function during head regeneration in Schmidtea mediterranea. Neurotoxicol. Teratol. 48, 28–32.

Maes, J., Verlooy, L., Buenafe, O. E., de Witte, P. A. M., Esguerra, C. V and Crawford, A. D. (2012). Evaluation of 14 organic solvents and carriers for screening applications in zebrafish embryos and larvae. PLoS One 7, e43850.

Mannini, L., Rossi, L., Deri, P., Gremigni, V., Salvetti, A., Saló, E. and Batistoni, R. (2004). Djeyes absent (Djeya) controls prototypic planarian eye regeneration by cooperating with the transcription factor Djsix-1. Dev. Biol. 269, 346–59.

Mineta, K., Nakazawa, M., Cebria, F., Ikeo, K., Agata, K. and Gojobori, T. (2003). Origin and evolutionary process of the CNS elucidated by comparative genomics analysis of planarian ESTs. Proc. Natl. Acad. Sci. U. S. A. 100, 7666–71.

Mondoux, M. A., Love, D. C., Ghosh, S. K., Fukushige, T., Bond, M., Weerasinghe, G. R., Hanover, J. A. and Krause, M. W. (2011). O-linked-N-acetylglucosamine cycling and insulin signaling are required for the glucose stress response in Caenorhabditis elegans. Genetics 188, 369–82.

Pagán, O. R., Rowlands, A. L. and Urban, K. R. (2006). Toxicity and behavioral effects of dimethylsulfoxide in planaria. Neurosci. Lett. 407, 274–278.

Parng, C., Roy, N. M., Ton, C., Lin, Y. and McGrath, P. (2007). Neurotoxicity assessment using zebrafish. J. Pharmacol. Toxicol. Methods 55, 103–12.

Parzefall, W. (2008). Minireview on the toxicity of dietary acrylamide. Food Chem. Toxicol. 46, 1360–4.

Peterson, R. T., Nass, R., Boyd, W. A., Freedman, J. H., Dong, K. and Narahashi, T. (2008). Use of non-mammalian alternative models for neurotoxicological study. Neurotoxicology 29, 546–55.

Rajini, P. S., Melstrom, P. and Williams, P. L. (2008). A Comparative Study on the Relationship Between Various Toxicological Endpoints in Caenorhabditis elegans Exposed to Organophosphorus Insecticides. J. Toxicol. Environ. Health. A 71, 1043–1050.

Reddien, P. W. (2013). Specialized progenitors and regeneration. Development 140, 951–7.

Reddien, P. W. and Sánchez Alvarado, A. (2004). Fundamentals of planarian regeneration. Annu. Rev. Cell Dev. Biol. 20, 725–57.

Richendrfer, H., Pelkowski, S. D., Colwill, R. M. and Créton, R. (2012). Developmental sub-chronic exposure to chlorpyrifos reduces anxiety-related behavior in zebrafish larvae. Neurotoxicol. Teratol. 34, 458–65.

Rink, J. C. (2013). Stem cell systems and regeneration in planaria. Dev. Genes Evol. 223, 67–84.
Roh, J.-Y. and Choi, J. (2008). Ecotoxicological evaluation of chlorpyrifos exposure on the nematode Caenorhabditis elegans. Ecotoxicol. Environ. Saf. 71, 483–9.

Sakurai, T., Lee, H., Kashima, M., Saito, Y., Hayashi, T., Kudome-Takamatsu, T., Nishimura, O., Agata, K. and Shibata, N. (2012). The planarian P2X homolog in the regulation of asexual reproduction. Int. J. Dev. Biol. 56, 173–82.

Scimone, M. L., Kravarik, K. M., Lapan, S. W. and Reddien, P. W. (2014). Neoblast Specialization in Regeneration of the Planarian Schmidtea mediterranea. Stem Cell Reports 3, 339–352.

Selderslaghs, I. W. T., Van Rompay, A. R., De Coen, W. and Witters, H. E. (2009). Development of a screening assay to identify teratogenic and embryotoxic chemicals using the zebrafish embryo. Reprod. Toxicol. 28, 308–20.

Sipes, N. S., Padilla, S. and Knudsen, T. B. (2011). Zebrafish—As an integrative model for twenty-first century toxicity testing. Birth Defects Res. Part C Embryo Today Rev. 93, 256– 267.

Sprando, R. L., Olejnik, N., Cinar, H. N. and Ferguson, M. (2009). A method to rank order water soluble compounds according to their toxicity using Caenorhabditis elegans, a Complex Object Parametric Analyzer and Sorter, and axenic liquid media. Food Chem. Toxicol. 47, 722–8.

Stevens, A. S., Pirotte, N., Plusquin, M., Willems, M., Neyens, T., Artois, T. and Smeets, K. (2014). Toxicity profiles and solvent-toxicant interference in the planarian Schmidtea mediterranea after dimethylsulfoxide (DMSO) exposure. J. Appl. Toxicol. 35, 319–326.

Talbot, J. and Schötz, E.-M. (2011). Quantitative characterization of planarian wild-type behavior as a platform for screening locomotion phenotypes. J. Exp. Biol. 214, 1063–7.

Tang, J., Cao, Y., Rose, R. L., Brimfield, A. A., Dai, D., Goldstein, J. A. and Hodgson, E. (2001). Metabolism of chlorpyrifos by human cytochrome P450 isoforms and human, mouse, and rat liver microsomes. Drug Metab. Dispos. 29, 1201–4.

Truong, L., Reif, D. M., St Mary, L., Geier, M. C., Truong, H. D. and Tanguay, R. L. (2014). Multidimensional in vivo hazard assessment using zebrafish. Toxicol. Sci. 137, 212–33.

Umesono, Y., Tasaki, J., Nishimura, Y., Hrouda, M., Kawaguchi, E., Yazawa, S., Nishimura, O., Hosoda, K., Inoue, T. and Agata, K. (2013). The molecular logic for planarian regeneration along the anterior-posterior axis. Nature 500, 73–6.

Vliet, E. van (2011). Current Standing and Future Prospects for the Technologies Proposed to

Transform Toxicity Testing in the 21st Century. ALTEX 28,.

Wagner, D. E., Wang, I. E. and Reddien, P. W. (2011). Clonogenic neoblasts are pluripotent adult stem cells that underlie planarian regeneration. Science 332, 811–6.

Watson, F. L., Schmidt, H., Turman, Z. K., Hole, N., Garcia, H., Gregg, J., Tilghman, J. and Fradinger, E. A. (2014). Organophosphate pesticides induce morphological abnormalities and decrease locomotor activity and heart rate in Danio rerio and Xenopus laevis. Environ. Toxicol. Chem. 33, 1337–45.

Yu, X., Zhao, W., Ma, J., Fu, X. and Zhao, Z. J. (2011). Beneficial and harmful effects of alcohol exposure on Caenorhabditis elegans worms. Biochem. Biophys. Res. Commun. 412, 757–62.

Yuan, Z., Zhao, B. and Zhang, Y. (2012). Effects of dimethylsulfoxide on behavior and antioxidant enzymes response of planarian Dugesia japonica. Toxicol. Ind. Health 28, 449– 457.

Chapter 3. Multi-behavioral endpoint testing of an 87-chemical compound library in freshwater planarians

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Abstract

There is an increased recognition in the field of toxicology of the value of medium-to-high-throughput screening methods using *in vitro* and alternative animal models. We have previously introduced the asexual freshwater planarian *Dugesia japonica* as a new alternative animal model and proposed that it is particularly well-suited for the study of developmental neurotoxicology. In this paper, we discuss how we have expanded and automated our screening methodology to allow for fast screening of multiple behavioral endpoints, developmental toxicity, and mortality. Using an 87-compound library provided by the National Toxicology Program (NTP), consisting of known and suspected neurotoxicants, including drugs, flame retardants, industrial chemicals, polycyclic aromatic hydrocarbons (PAHs), pesticides and presumptive negative controls, we further evaluate the benefits and limitations of the system for medium-throughput screening, focusing on the technical aspects of the system. We show that, in the context of this library, planarians are the most sensitive to pesticides with 16/16 compounds causing toxicity and the least sensitive to PAHs, with only 5/17 causing toxicity. Furthermore, while none of the presumptive negative controls were bioactive in adult planarians, 2/5, acetaminophen and acetylsalicylic acid, were bioactive in regenerating worms. Notably, these compounds were previously reported as developmentally toxic in mammalian studies. Through parallel screening of adults and developing animals, planarians are thus a useful model to detect such developmental-specific effects, which was observed for 13 chemicals in this library. We use the data and experience gained from this screen to propose guidelines for best practices when using planarians for toxicology screens.

Introduction

It has been nearly a decade since the launch of the "Toxicology Testing in the 21st century" (Tox21; www.tox21.gov) federal initiative to transform toxicology testing in the United States. Its ongoing goal is to dramatically increase the coverage of chemical testing by replacing traditional mammalian models with alternative testing strategies amenable to high-throughput screening (HTS) (Collins et al., 2008). Since its inception, thousands of chemicals have been screened *in vitro* using HTS robotic systems to identify mechanisms of action and prioritize chemicals for further targeted testing. However, connecting those HTS data to their *in vivo* relevancy to be predictive of effects on human health remains challenging as important aspects of biology, such as xenobiotic metabolism and interactions between cell types, are inherently missing in these *in vitro* systems. In addition, although these assays often focus on key molecular and cellular targets underlying known toxicity pathways, more knowledge is needed to connect these molecular and cellular effects to functional consequences on organismal health to discern their significance. Realizing this need and the urgency of the matter, the development of medium-throughput screening (MTS)-amenable alternative animal models, such as zebrafish and nematodes, was encouraged as part of the Tox21 initiative. These animal models are attractive MTS toxicology systems due to their ease of breeding and chemical administration, low cost, small size, short developmental time, and genetic tractability (Boyd et al., 2012; Boyd et al., 2015; Hill et al., 2005; Tejeda-Benitez and Olivero-Verbel, 2016; Truong et al., 2014). Moreover, each system provides unique advantages. For example, the transparency of zebrafish larvae, which develop externally, allows for a breadth of morphological assessments of the development of internal structures in living animals (Kimmel et al., 1995; Truong et al., 2014). However, despite these advantages, the toxicology community remains divided on the added value of these

alternative systems, particularly as each has its own drawbacks, species-specific sensitivities and discrepancies with humans, as with any system (Boyd et al., 2015; Scholz, 2013).

A battery approach using multiple complementary testing platforms allows for comparative analyses to find concordance between systems and produce more weight of evidence for reliable and relevant predictions of effects on human health, as demonstrated by a recent battery screen on organophosphorus flame retardants (Behl et al., 2015). These predictions can then be verified by targeted testing in mammalian models, which, although not without caveats, are still considered the gold standard in toxicology, particularly for regulatory decisions (Tsuji and Crofton, 2012).

We have previously introduced the freshwater planarian *Dugesia japonica* as a new alternative animal model for developmental neurotoxicology and shown that it possesses comparable sensitivity to other, more established alternative models (Hagstrom et al., 2015). In addition, the planarian system offers the unique advantage to study adult and regenerating/developing animals in parallel with the same assays, because in this asexual species the sole form of neurodevelopment is neuroregeneration of a head from a tail piece following fission. Finally, planarians have a large behavioral repertoire that can be quantified and assessed in a fully automated fashion, providing multiple distinct endpoints of neuronal function. Importantly, the planarian nervous system contains most of the same neurotransmitters as the mammalian brain and is considered more structurally similar to the vertebrate brain than other invertebrate brains (Buttarelli et al., 2008; Cebrià, 2007; Mineta et al., 2003; Ross et al., 2017; Umesono et al., 2011). A brief review of the planarian nervous system and of neuroregeneration can be found in Supplementary Information, Section 1. Moreover, we have recently reviewed the history, challenges and benefits of planarians as a model for neurotoxicology (Hagstrom et

al., 2016).

While our previous work demonstrated the potential of *D. japonica* for toxicology screens, it was limited in scope (10 compounds, including controls) (Hagstrom et al., 2015). Most of the experiments and analysis were conducted manually, which limited throughput and scalability. Our screening platform has since been greatly expanded and optimized to incorporate more behavioral endpoints that are all assayed in a fully automated fashion.

In this study, we evaluate the capabilities and limitations of this improved planarian MTS platform by testing a library of 87 compounds provided by the National Toxicology Program (NTP), consisting of known and suspected developmental neurotoxicants and negative controls. This compound library, which has also been tested in other alternative systems, including zebrafish and *in vitro* cell culture systems (see other articles in this special issue), gives us a unique opportunity to test the robustness and relevancy of the planarian system as a whole and of the specific endpoints we have developed to assay different neuronal functions. We focus on evaluating the technical aspects of our expanded screening platform and the utility of the planarian model system for toxicology screens, setting clear standards and challenges that need to be addressed for the field going forward. A direct comparison of the results of this planarian screen with a zebrafish model, and with available mammalian data, are the focus of a companion paper in this Special Issue (Hagstrom et al.).

Material and methods

Test animals: Freshwater planarians of the species *D. japonica,* originally obtained from Shanghai University, China, and cultivated in our lab >5 years, were used for all tests. Planarians were stored in 1x Instant Ocean (IO, Blacksburg, VA) in Tupperware containers at 20°C in a Panasonic refrigerated incubator in the dark. Animals were fed organic chicken or beef liver purchased from a local butcher twice a week. Planarian containers were cleaned 3 times a week per standard protocols (Dunkel et al., 2011). Animals were starved for at least 5 days before being used for experiments and their containers were cleaned immediately prior to worm selection for experiments. Test worms were manually selected to fall within a certain range of sizes and we found full worm length, after automated size measurement, to be 7.3mm +/- 2.3mm (mean +/- SD), and tail worm length to be 7.3mm +/- 2.7mm (mean +/- SD). Slightly larger intact planarians (~1-2 mm larger to account for the size of the head) were chosen for regenerating tail experiments such that the final sizes of the amputated tail pieces were similar to the full/adult test planarians. Some animals were recovered after the screen and reintroduced into the normal population after a minimum of 4 weeks of separate care. As planarians undergo dynamic turnover of all cell types within a few weeks (Rink, 2013) and as we observed no qualitative differences in behavior between recovered and wild-type animals, these recovered worms were considered functionally wild-type. For all experiments, only fully regenerated worms which had not been fed within one week and which were found gliding normally in the container were used. To study regenerating animals, on day 1, intact worms were amputated, by cutting posterior to the auricles and anterior to the pharynx with an ethanol-sterilized razor blade, no more than 3 hours before the compounds were added. During the course of the screen, some animals underwent fission producing at least 2 pieces (a head and a tail piece) (see below and

Supplementary Information, Section 4). To obtain full and tail worms of comparable size, we amputate slightly larger worms to obtain the tail pieces. Since fission probability increases with worm size (Carter et al., 2015; Yang et al., 2017) and decapitation (Bronsted, 1955; Hori and Kishida, 1998; Morita and Best, 1984), fission primarily occurred for tail worms. For these cases, only the head piece was considered in all morphological and behavioral analyses, as this would represent the first regenerated brain.

Test compounds: The 87-compound library (summarized in Supplementary Table 1) was provided by the NTP and included 5 categories: pesticides, flame retardants, drugs, industrial compounds and polycyclic aromatic hydrocarbons (PAHs) (Behl et al., 2018). Five negative controls were also included. The compounds were provided as ~20mM stocks (or lower) in 100% dimethyl sulfoxide (DMSO, Gaylord Chemicals, Slidell, LA) in a 96-well plate. The master library was stored at -80ºC.

Chemical preparation and screen setup: The 87-chemical library was separated into 5 "Chemical Sets" of 18 (sets 1-4) or 15 (set 5) chemicals (Supplementary Table 1). Chemicals in the same Chemical Set were tested on the same day, i.e. the same experiment. All chemicals, regardless of provided concentration, were treated the same. 0.5% DMSO was used as solvent control, because we have previously shown that there are no effects on planarian morphology or behavior at this concentration (Hagstrom et al., 2015). To keep the final DMSO concentration constant at 0.5%, the highest concentration tested in the screening process was a 200-fold dilution of the original provided chemical stock. Subsequent concentrations were a 10-fold dilution of the previous. Thus, each compound was tested at 5 concentrations, generally ranging from 10nM to 100μM (with some exceptions, see Supplementary Table 1). Each 48-well screening plate assayed n=8 planarians in a 0.5% DMSO control, and n=8 worms each per

concentration of chemical (5 test concentrations per plate in total) (Figure 3.1). Experiments were performed in triplicate (independent experiments performed on different days, final n=24) with the concentrations shifted down two rows (one row in run D, see raw data in the Dryad Digital Repository (doi: 10.5061/dryad.mk6m608)) with each replicate to control for edge effects. For each chemical and each experiment, 2 plates, one containing full (intact) planarians and one containing regenerating tails, were assayed. Screening was performed on day 7 and day 12.

Plate setup and storage: 200X stock plates of the tested chemicals were prepared ahead of time by transferring 50µl of the provided chemical stock into one well of a 48-well plate (Genesee, San Diego, CA). 10-fold serial dilutions were performed in DMSO in the same plate using a multi-pipettor to create the remaining stock concentrations. The control well contained DMSO only. These plates were sealed with foil seals (Thermo Scientific, Waltham, MA) and stored at -20°C. On the day of plate set-up, the 200X stock plates were thawed at room temperature for approximately 30 minutes. 10X stocks plates were then made by diluting the 200X stocks 20X in IO water. Dilutions were mixed by rotation on an orbital shaker for approximately 10 minutes before use. The highest concentration of some chemicals, noted in Supplementary Table 1, precipitated out of solution in the 10X stock plates due to low solubility in water.

Screening plates were prepared by transferring individual full planarians or amputated tail pieces into the wells of a 48-well plate with 200µl of IO water using a P1000 pipet with a cut-off tip. A multi-pipettor was used to remove 20µl of IO water from each well and add 20µl of the appropriate 10X stock solution. The plates were sealed with ThermalSeal RTS seals

(Excel Scientific, Victorville, CA) to prevent evaporation and gas exchange with the environment. The plates were stored, without their lids, in stacks in the dark at room temperature when not being screened. Prepared plates were only moved to the screening platform when screened at day 7 and day 12.

Screening platform: We have further automated and expanded the custom-built planarian screening platform introduced in (Hagstrom et al., 2015). The new platform consists of a commercial robotic microplate handler (Hudson Robotics, Springfield Township, NJ), two custom-built imaging systems and multiple assay stations (Figure 3.1). One imaging system is specifically used to image individual planarians at high spatial resolution to allow for quantification of lethality, morphology and eye regeneration. It consists of 4 monochromatic Flea USB3 cameras (FLIR Systems Inc., Wilsonville, OR), each equipped with a fixed-focal (16mm) optical lens (Tamron, Saitama, Japan) and 5mm spacer (Edmund Optics, Santa Monica, CA). Each camera is used to image a single well, thus 4 wells are imaged simultaneously and the entire plate is scanned in the x- and y- directions. The second imaging system consists of one monochromatic Flea USB3 camera, equipped with a fixed-focal (25mm) double-gauss lens (Edmund Optics) and red filter (Roscolux, Stamford, CT), which is used to image the whole plate from above for all behavioral assays. To prevent angular distortion on the edge of the wells, a Fresnel lens (MagniPros, South El Monte, CA) is placed on top of the plate when imaging with the single camera. All cameras are mounted on a custom rail platform (Inventables Inc., Chicago, IL), which enables x-, y- and linear motion. All assays were imaged at a frame rate of 5 frames per second . Different assay stations were designed specifically for different assays, as explained below. The imaging systems, assay stations and plate handler were controlled by the computer. The stimuli and illuminations in the assays were mainly controlled via Arduino (Arduino,

Somerville, MA). Image acquisition was controlled through custom LabVIEW scripts. All assays were performed in the following order, whereby the notation in brackets indicates on which day(s) the assay was performed: phototaxis (d7/d12), unstimulated locomotion (d7/d12), lethality/regeneration (d7/d12), thermotaxis (d12) and scrunching (d12) (see also Figure 3.1). Any data analysis which had to be cross-checked manually was performed blinded by a single investigator, who was not given the chemical identity of the plates. The raw data are provided in the Dryad Digital Repository (doi:10.5061/dryad.mk6m608).

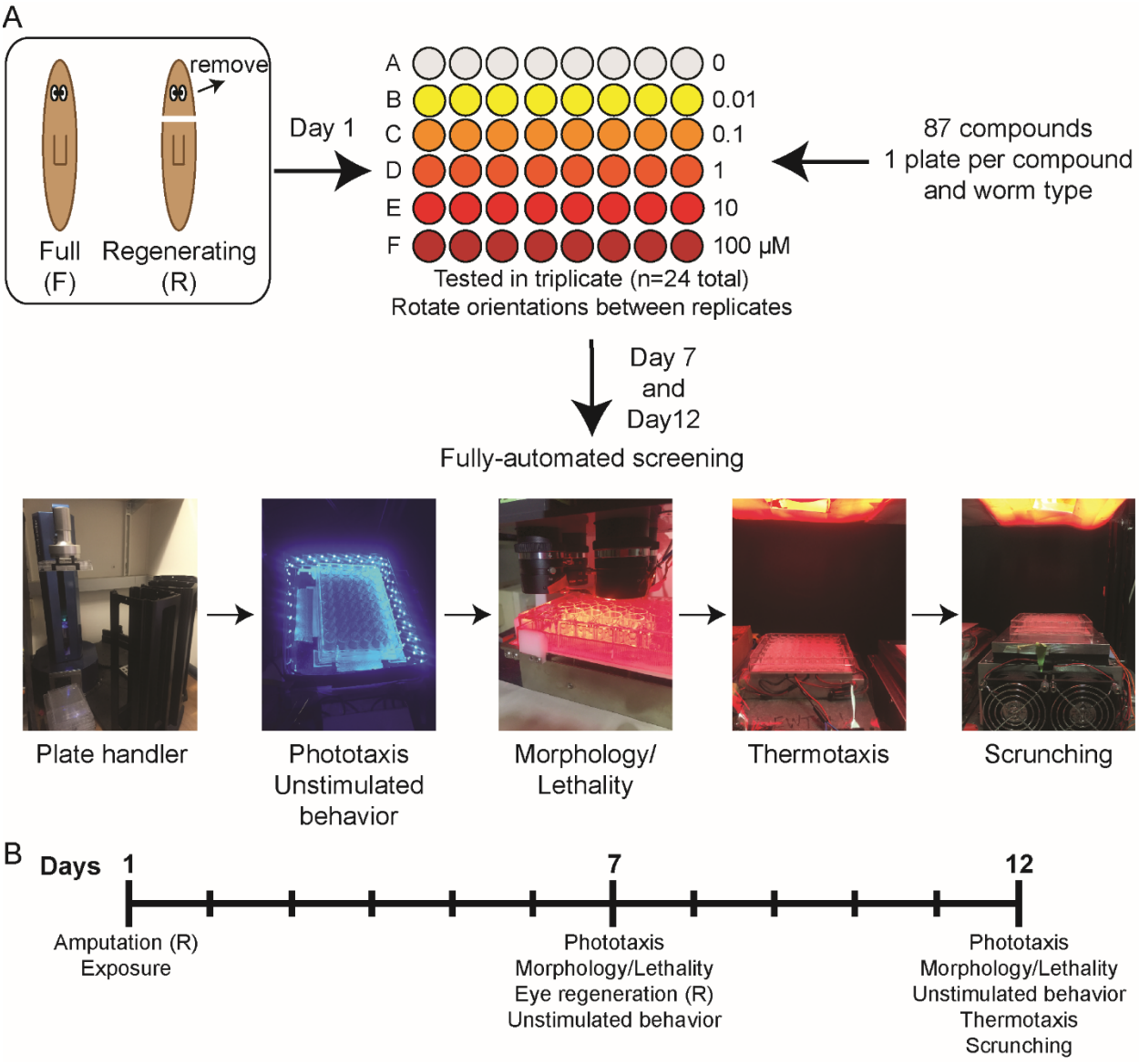


Figure 3.1. Overview of planarian screening platform. (A) Schematic of screening workflow. On day 1, for each chemical, one plate each is filled with either full planarians (F) or regenerating tail pieces (R). 5 test concentrations and 1 control concentration (0.5% DMSO) are placed in each row with n=8 animals per concentration. Plate orientation is altered between replicates. Screening is performed on days 7 and 12. (B) The timeline shows which assays are performed on which screening days.

Lethality assay: To assay planarian lethality and eye regeneration, high-resolution imaging of each individual well was performed. Since planarians tend to rest on the edge of the well, prior to imaging each set of 4 wells, the screening plate was placed on a microplate orbital shaker (Big Bear Automation, Santa Clara, CA) and shaken for 1 second at 800 rotations per minute (rpm) to force the worms to the center of the well. Each well was then imaged for 10 seconds. The plate was illuminated from above by red LED strings (Amazon, Seattle, WA) attached around the camera lens.

Semi-automatic analysis was performed on the image sequence of each single planarian to determine whether the animal was alive or dead. Death was determined by the absence of the worm or the presence of a disintegrating body, using the fact that a dead planarian usually disintegrates (Buchanan, 1935). An alive planarian was marked as '0'and a dead one as '1' (Figure 3.2A-B). If the worm "suicides" by leaving the water and thus drying out, the respective well would be marked as '10' and discarded in the data analysis. Lethality was calculated as

$$lethality = \frac{total\ number\ of\ dead\ planarians}{total\ number\ of\ planarians}$$

Where "total number of planarians" excludes any suicides. For compounds which showed significant lethality in the concentration range tested (see Statistical Testing section below), the fraction of dead planarians as a function of concentration at days 7 and 12 was fitted as described in (Hagstrom et al., 2015) using a Hill equation to obtain the LC_{50} (Supplementary Figure S1). Of note, fissioned planarians in a single well were marked as one unit. If any fissioned piece was alive in one well, this well was considered to contain an alive worm and marked as '1'.

Eye regeneration assay: Eye regeneration data was also collected from the high-resolution imaging performed in the lethality assay (described above). Image analysis was

performed with a custom Python-based machine learning algorithm using a transfer learning neural network (Pan and Yang, 2010). A custom pre-processing program was used in Python to crop 100 x 100 pixel^2 images of a planarian's head region from the original images. The cropped images were imported into the neural network, which categorized the worms based on a prediction of the number of eyes in the images: normal (2 eyes), abnormal (0, 1 eye or >2 eyes), and invalid (for example, when the worm was on the edge of the well, flipped over, or the head region was not properly cropped) (Figure 3.2D-G). The neural network was trained using a training set consisting of 2206 images of normal eyes, 1047 images of abnormal eyes and 6703 images with undetectable quality. The training set was labeled semi-manually with a customized computer program. The prediction results of each image for each alive planarian were integrated using a custom MATLAB script to make the final decision of the number of eyes in this regenerating animal. If more than 1 image frame predicted normal eyes, the planarian was determined to have normal eyes. If more than 1 image frame predicted abnormal eyes, but no image frame predicted normal eyes, the worm was determined to have abnormal eyes. In all other cases, the image sequence was an invalid case, due to lack of analyzable images resulting from worm positioning in the well which obscured the eyes, see Figure 3.2G), and discarded in the following analysis. Since the prediction of the "abnormal" category was often inaccurate because of the small training set and large variability in data, we manually double checked all results predicted to be "abnormal" and invalid. For planarians which underwent fission during the course of the screen, resulting in more than 1 animal in a well, the number of regenerated eyes in the head piece was scored manually. The eye regeneration rate was calculated as

$$eye\ regeneration\ rate = \frac{number\ of\ planarians\ with\ 2\ eyes\ regenerated}{number\ of\ analyzable\ planarians}$$

Unstimulated behavioral assay: As planarians tend to rest when stored in the dark, screening plates were firstly shaken for 6 seconds at 900rpm on the microplate shaker used in the lethality assay, to encourage motion before imaging. The screening plate was then moved by the automatic plate handler onto a transparent plate holder. There it was imaged for 3 min by the single camera, with a cold LED panel (B&H Photo Video, New York, NY) equipped with a red filter (Roscolux, Stamford, CT) placed under the transparent plate holder to provide illumination for tracking.

Image analysis was performed using a custom MATLAB script, based on center of mass (COM) tracking. To accurately determine the COM of each planarian, the tracking analysis was specifically optimized for fissioned worms (see Supplementary Information, Section 4). This assay provided 2 readouts: the fraction of time spent resting and the instantaneous speed of locomotion. The instantaneous speeds were calculated for all tracks over 2-second intervals to increase the signal-to-noise ratio (Hagstrom et al., 2015). An empirically determined absolute speed cutoff was used to distinguish the planarians' moving and resting behaviors (see Supplementary Information, Section 5). Instantaneous speeds less than 0.5 mm/s were considered to represent resting and were disregarded in speed calculations. The fraction of time spent resting was calculated as the amount of time resting divided by the total time tracked. Speed values > 0.5 mm/s represent planarian locomotion and were averaged to calculate the mean speed for each planarian. Of note, this speed includes instances of both swimming and gliding behaviors and thus differs from our previously used measure ((Hagstrom et al., 2015), Supplementary Information, Section 5). Planarians with no tracking data (i.e. tracking was lost for worms moving at the edge of the well due to low contrast) were considered non-analyzable and excluded for further analysis. In <4% of day 7 plates and <12% of day 12 plates (full animal

and regenerating tails), 1 or 2 animals were non-analyzable. In ~1% of the day 12 plates, 3-5 animals were excluded. For fissioned worms, when the head and tail pieces were distinguishable, analysis was only performed on the head piece. Otherwise, when the head and tail pieces were indistinguishable, analysis was only performed on the fastest piece, as heads generally move faster.

Phototaxis: For this assay, the same transparent plate holder was used as for the unstimulated behavioral assay. Planarians are negative phototactic to blue light and insensitive to red light (Paskin et al., 2014). To study negative phototactic behavior, blue LED lights (SuperNight, Portland, OR) surrounding the screening plate were used to provide the blue light stimulus. Additionally, red backlighting underneath the plate holder provided light for tracking throughout the assay. Similar to photomotor response studies in zebrafish larvae (Kokel and Peterson, 2011; Truong et al., 2014), we used a combination of dark-light-dark-light cycles. First, the plate was imaged for 30 seconds using red light (dark condition) and then imaged for 30 seconds with both red and blue lights (light condition) (Figure 3.5A). This sequence was then repeated. The red filter on the single camera blocks the blue light, which optimizes the imaging of this assay. Because it was only found after screening was complete that the second dark cycle was too short for animals to adapt, we compared the planarians' behavior in the first dark cycle with that in cycles 2-4 (1^{st} light cycle, 2^{nd} dark cycle and 2^{nd} light cycle) instead of analyzing each dark/light cycle sequence separately.

Image analysis was automated using a custom MATLAB script. The instantaneous speeds were calculated as in the unstimulated assay. The instantaneous speed was averaged in cycle 1 and cycles 2-4. Any average speed value < 0.01 mm/s (background noise level) was set

to 0.01 mm/s. Speed cutoffs were set as the mean speed of the control populations in DMSO measured in the unstimulated behavioral assay, for Day 7/Day 12 full worms and regenerating tails. In the test concentrations, planarians with a mean speed in cycle 1 lower than the speed cutoff were excluded due to their relatively high background activity, which would cause false positives in the phototaxis assay. Otherwise, the mean speed in cycles 2-4 was normalized by the mean speed in cycle 1 (background activity). Planarians with a normalized mean speed in cycles 2-4 higher than 1 were defined as having reacted to the light stimulus, and marked as "1". If the normalized mean speed in cycles 2-4 did not exceed 1, the planarian was considered to have no reaction, and marked as "0". If the planarian was dead or had high background activity, it was discarded and marked as "NaN". The phototaxis response rate was calculated as

$$phototaxis\ response\ rate = \frac{total\ number\ of\ worms\ reacting\ to\ light}{total\ number\ of\ analyzable\ worms}$$

Thermotaxis assay: The plate was placed on a custom setup with 12 peltiers (15mm x 15mm) (Digi-key, Thief River Falls, MN) that are evenly spaced and embedded in an aluminum heat sink. The peltiers are arranged in a matrix of 3 rows x 4 columns (i.e. 4 wells share one peltier) and powered by an AC to DC power supply (Genssi, Las Vegas, NV) (Figure 3.5B). This setup, which is controlled automatically through an Arduino board, creates an identical heat gradient with a temperature difference of 3-4°C in each well of the screening plate. During the assay, the plate was imaged without the heat gradient (ambient temperature) for 2 minutes, and then imaged with the heat gradient for 4 minutes by the single camera. The plate was illuminated from the top by a custom-made red LED ceiling light which does not obscure the view of the camera.

Image analysis was performed using a custom, automated MATLAB script. The COM of each planarian was tracked over time and used to calculate the fraction of time the animal spent in the cold area in the well when the gradient is on. Since it takes time to establish a stable heat gradient across the well, we only accounted for the fraction of time the worm spent in the cold area during the last two minutes of the assay. The cold area in each well was defined as the area of a sector with central angle of 120° (Figure 3.5B-D). Since the image analysis worked poorly on fissioned planarians, since it expects one object per well, we manually calculated the fraction of time the head piece spent in the cold area.

Scrunching assay: Scrunching is a musculature-driven escape gait in planarians, which can be triggered by multiple external stimuli, including amputation, high heat, electric shock and low pH. It is characterized by asymmetric elongation-contraction cycles (with elongation time > contraction time), and a species-specific frequency and amplitude (Cochet-Escartin et al., 2015). To induce scrunching in the screening platform, the screening plate was placed on a peltier plate (TE Technology Inc., Traverse City, MI), which was controlled by the computer through a temperature controller board (TE Technology Inc.), to increase the aquatic temperature in the wells. The temperature of the peltier plate was initially set to 65°C for the first 30 seconds to quickly heat up the plate from room temperature. Then, the temperature was gradually decreased to 43°C to stabilize the aquatic temperature across the plate at around 32°C for 4 minutes (Supplementary Figure S3), which was sufficient to induce wild-type *D. japonica* to scrunch. The plate was imaged by the single camera and illuminated by the same type of custom red LED light used in thermotaxis (see above).

Image analysis was performed using a custom, automated MATLAB script. The COM and length of each planarian were tracked over time. The worm's length over time was plotted and smoothed to detect instances of scrunching. We extracted body length oscillations in the smoothed plot which fulfilled the scrunching criteria mentioned above (asymmetric cycles, characteristic frequency) to determine instances of scrunching (Figure 3.5C). We defined such oscillations consisting of >3 consecutive peaks in the body length versus time plot as scrunching and marked the planarian as "1". If no such characteristic oscillations were found, the worm was marked as "0" for no scrunching. If the planarian was dead or not properly detected (not enough tracking data), it was discarded and marked as "NaN". The automated image analysis was not possible with fissioned planarians and thus these animals were scored manually. Scrunching rate was calculated as

$$scrunching\ rate = \frac{total\ number\ of\ scrunching\ worms}{total\ number\ of\ analyzable\ worms}$$

Statistical testing: All data from the triplicate runs were compiled before performing any statistical test. For lethality, eye regeneration, phototaxis and scrunching endpoints, significant effects were determined using a one-tailed Fisher's exact test to compare the rates determined for each chemical concentration with the rate of its own DMSO controls. For thermotaxis and unstimulated behavioral endpoints, Tukey's interquartile test was first used to remove any outliers, with at most 5% (e.g. 1 out of 24 worms) of the data removed. Since the distribution of the thermotaxis data was highly skewed and variable, a non-parametric one-tailed Mann Whitney U-test was used to compare the distributions of the fraction of time in the cold area for each chemical concentration with the respective distribution of its own control. For speed and fraction of time resting from the unstimulated behavior assay, Lilliefors test was first used to test the normality of the samples. Depending on whether the sample distributions were normal or not,

we performed either a parametric two-tailed t-test or a nonparametric two-tailed Mann-Whitney U-test, respectively. For all endpoints, any condition with a p-value less than 0.05 was considered statistically different from the controls. However, we observed that due to low variance in some individual plate control populations (and high variability across plates), some statistically relevant hits were likely not biologically meaningful (see Supplementary Information Section 2 and Supplementary Figure S6). Examples such as this resulted in a large number of dose-independent hits and hits in the negative controls, together suggesting these may be false positives. Thus, to reduce potential false positives, we disregarded hits that had a smaller effect than determined by a "biological relevance" cutoff based on the variability of the DMSO controls in each assay. These cutoffs were meant to disregard hits that fell within the variability of the DMSO controls across all plates and were thus based on the distribution of the compiled control values for each chemical (n=87) and endpoint (Supplementary Figure S4). High variability within animal behavior endpoints has also been observed in zebrafish (Zhang et al., 2017). For endpoints where the distribution of the compiled control values was normal (unstimulated behavior and phototaxis), cutoffs were based on mean +/- 2 or 3 SD (see Supplementary Information), respectively. For endpoints where the distribution of the compiled control values was not normal (day 12 lethality, thermotaxis, and scrunching), cutoffs were set as the 5^{th} and 95^{th} quantiles. These cutoffs were empirically determined to encompass the variability of the DMSO controls and to minimize dose-independent hits (see Supplementary Information Section 2 for more details). Similar approaches to creating assay-specific noise threshold levels has been described previously (Behl et al., 2015). Of note, the distributions of control values in the day7 lethality and eye regeneration endpoints were so narrow (Supplementary Figure S4) that biological relevancy cutoffs were not appropriate. However, because controls exhibited few

deaths at day 7, some chemical concentrations were designated as statistically significant hits for day 7 lethality but not day 12. These cases were excluded as artifacts. Moreover, we checked for inconsistency in the data to find instances where a single plate was responsible for designating a "hit". Inconsistent hits were defined as instances with only 1 replicate outside of the biological relevancy cutoff range and two replicates within the control variability. These hits were therefore excluded (see Supplementary Figure S5 for the statistical workflow). Other groups have reportedly dealt with similar issues with plate-to-plate variability by rerunning inconsistent plates (Zhang et al., 2017), whereas we have decided to keep all data. The lowest observed effect level (LOEL) was determined as the lowest concentration which showed a significant effect (i.e. statistically significant and passed inconsistency and biological relevancy tests, Supplementary Figure S5) in any endpoint. All statistical analyses were performed in MATLAB (see Table 3.1 for a summary).

To determine the observed power of each of the tested endpoints, we performed post-hoc power analysis using G*power (Faul et al., 2007) (Table 3.1). For some endpoints our distributions were highly skewed and/or multi-modal (unstimulated behavior and thermotaxis assays) and we were unable to transform them into normal distributions. Thus, in these cases power analysis could not be performed, since G-power expects a normal distribution as input.

Table. 3.1. Summary of statistical testing

Assay	Endpoints	Statistical test	Median observed power
Lethality	Lethality rate	One-tailed Fisher's exact test	1
Morphology	Eye regeneration rate	One-tailed Fisher's exact test	0.99
Unstimulated behavior	Speed	Two-tailed T-test or Mann Whitney U-test	N/D*
	Fraction of time resting	Two-tailed T-test or Mann Whitney U-test	N/D*
Phototaxis	Phototaxis response rate	One-tailed Fisher's exact test	0.75
Thermotaxis	Fraction of time in cold area	One-tailed Mann Whitney U-test	N/D*
Scrunching	Scrunching rate	One-tailed Fisher's exact test	0.98

* N/D: not determined

Results

To evaluate the strengths and weaknesses of the planarian system for toxicology MTS, we screened an 87-compound library, provided by the NTP, consisting of known and suspected developmental neurotoxicants and five negative controls (Supplementary Table 1). Each chemical was tested at 5 concentrations, generally ranging from 10nM to 100μM, in both full (intact) planarians and regenerating tail pieces (n=8 each) (Figure 3.1), with a 0.5% DMSO solvent control population (n=8) in each plate. Six chemicals (BDE-153, Chrysene and Dibenz(a,h)anthracene, Bis(tributyltin) oxide, Benzo[g,h,i]perylene, and 2,3,7,8-Tetrachlorodibenzo-p-dioxin) were provided at lower than 20mM due to low solubility in DMSO and were thus tested at lower concentrations (see Supplementary Table 1 for concentrations). On day 7, when regenerating animals start to develop their photosensing system and regain motility (Hagstrom et al., 2015; Inoue et al., 2004), adult and regenerating planarians were assessed for viability, regeneration, locomotion and phototactic behavior. On day 12, all of these endpoints, except for regeneration, were tested again. In addition, on day 12, we evaluated the effects on two more stimulated behaviors: thermotaxis and scrunching. Screening on both days 7 and 12 allows us to evaluate the temporal dynamics of possible subchronic toxic effects and effects on regeneration (Figure 3.1). Raw data are available from the Dryad Digital Repository (doi: 10.5061/dryad.mk6m608).

Lethality and morphology

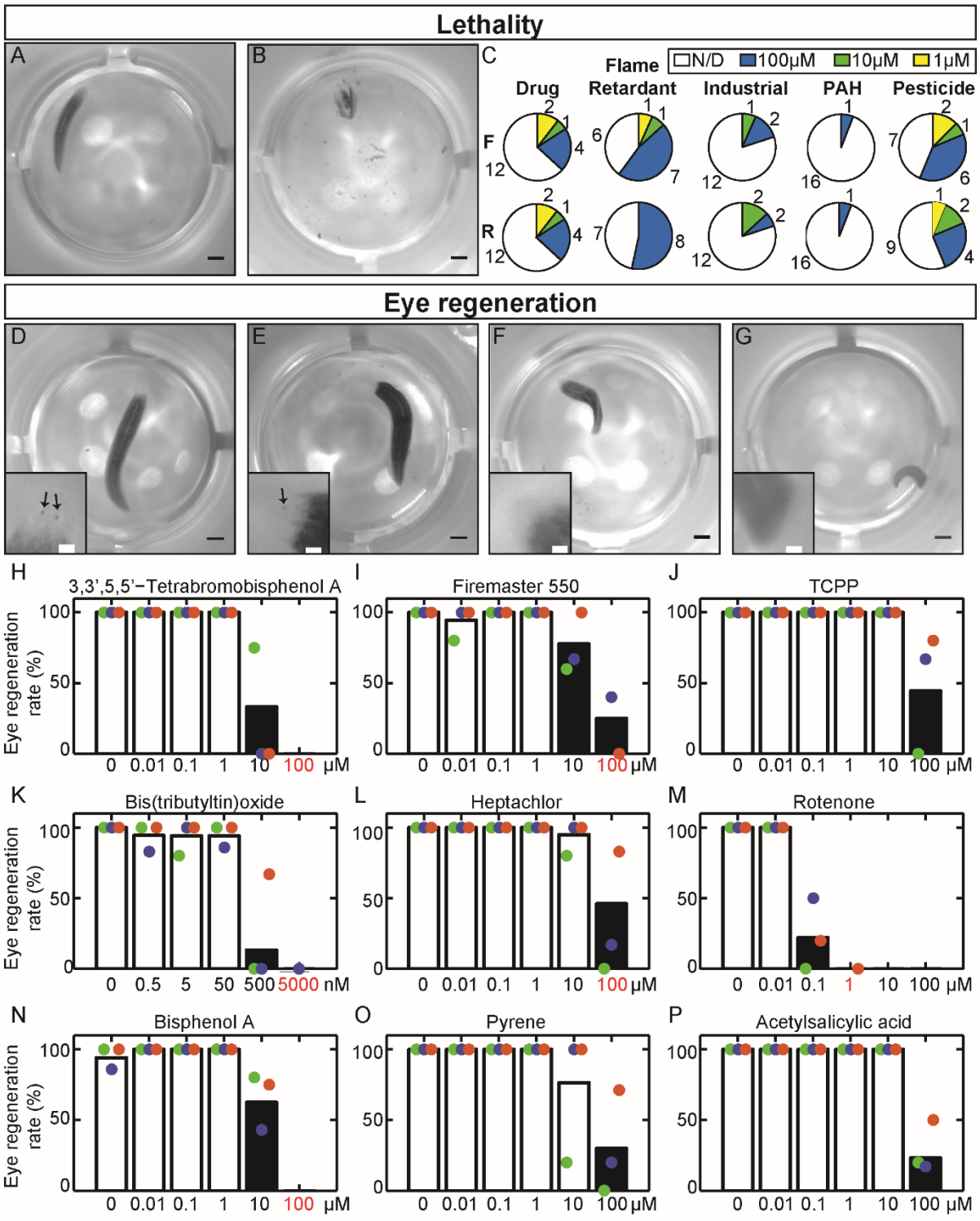
To evaluate whether the chemicals have an effect on planarian viability (Figure 3.2A-B), we performed statistical tests for all chemicals and, when appropriate, calculated the LC_{50} for chemicals with significant lethality (Supplementary Figure Sl and Supplementary Table 2). Over

the entire 12 days of screening, 29 of the 87 tested chemicals (33%) were significantly lethal for at least one concentration, with 27 of them already being lethal by Day 7. No significant lethality was found in any of the negative controls at the tested concentrations. While lethality was found in at least one chemical from each chemical class tested, the majority of lethal compounds (18 of 29, 62%) consisted of either flame retardants or pesticides (9 lethal chemicals each). As there are only 15 or 16 chemicals comprising each of these classes in the library, respectively, this also means that the majority of the chemicals in these classes (56-60%) were lethal to planarians. Full worms tended to be more sensitive to the lethal effects of some chemicals, as 6 chemicals caused significant day 12 lethality at lower concentrations in full worms than in regenerating tails. This difference was the most striking with the flame retardant 3,3',5,5'-Tetrabromobisphenol A as significant lethality was observed in full planarians at 1μM but in regenerating tails at 100μM. We attribute this difference in sensitivity of full and tail worms, which was also observed in a previous screen (Hagstrom et al., 2015), partially to the generally lower motility and potentially lower level of metabolism in regenerating tail pieces. In contrast, only two chemicals, the drug Diazepam and the industrial chemical Auramine O had lower day 12 lethality LOELs in regenerating tails than in full animals.

Eye regeneration was categorized as normal (2 eyes), abnormal (0 or 1 eye) or invalid (could not be analyzed) (Figure 3.2D-G). 21 chemicals (~24%) showed significant defects in eye regeneration. In the majority of these chemicals (12 of 21), regeneration defects may have been a consequence of overt systemic toxicity as effects occurred at day 12 significantly lethal concentrations (Figure 3.7). However, 9 of these 21 chemicals showed selective effects with the eye regeneration LOEL being less than that of the day 12 tail lethality LOEL. These selective

chemicals consisted of 3 pesticides, 3 flame retardants, 1 industrial chemical, 1 PAH, and 1 negative control (Acetylsalicylic acid, Figure 3.2H-P).

Figure 3.2. Lethality and morphology endpoints. High-resolution imaging of each well was used to determine whether a planarian was (A) alive or (B) dead. (C) Distributions of lethal chemicals and their day 12 LOEL by chemical class for full worms (F, top row) and regenerating tails (R, bottom row). Chemicals which were not found to be lethal at the tested concentrations are marked as N/D for "not determined". (D-F) High-resolution imaging of day 7 regenerating tails was used to evaluate whether the eyes had regenerated. A custom neural network was used to automatically detect whether the planarian had (D) 2 eyes (normal), or abnormal eyes, (either (E) 1 eye or (F) no eyes) as described in Materials and Methods. Insets show cropped and zoomed-in head regions. Arrows point to the eyes. (G) In some cases, it was impossible to correctly determine the number of eyes. Such cases were classified as invalid and discarded in the analysis. Black scale bars: 1mm. White scale bars: 0.2mm. (H-P) Eye regeneration rate (percentage of planarians with 2 regenerated eyes) shown for each replicate (dots) and for all combined data (bars) as a function of concentration for chemicals in which defects were seen in the absence of significant lethality. If no individual replicate data is shown, all animals were dead in this sample. Significant defects in eye regeneration are in black bars. Concentrations corresponding to the day 12 regenerating tail lethality LOELs for each chemical are in red text. No red text signifies no significant lethality was found in the range of concentrations tested. Chemicals shown are flame retardants (H) 3,3',5,5'-Tetrabromobisphenol A, (I) Firemaster 500 and (J) tris(2-Chloroisopropyl)phosphate (TCPP), pesticides (K) Bis(tributyltin)oxide, (L) Heptachlor and (M) Rotenone, (N) industrial Bisphenol A, (O) PAH Pyrene and (P) negative control Acetylsalicylic acid.


Lethality
A
B
C
N/D
100μM
10μM
1μM
Drug
Flame Retardant
Industrial
PAH
Pesticide
F
R
Eye regeneration
D
E
F
G
H
3,3',5,5'−Tetrabromobisphenol A
I
Firemaster 550
J
TCPP
K
Bis(tributyltin)oxide
L
Heptachlor
M
Rotenone
N
Bisphenol A
O
Pyrene
P
Acetylsalicylic acid
Eye regeneration rate (%)
0 0.01 0.1 1 10 100 μM
0 0.5 5 50 500 5000 nM

Unstimulated behavior

We evaluated whether the chemicals perturbed planarian unstimulated behavior by quantifying the worms' fraction of time resting and mean speed during the assay (Figure 3.3). Together, these endpoints demonstrate whether the exposed planarians were moving and if so, whether they were moving normally. Control animals, regenerating tails and full worms, were found to move at a mean speed of approximately 1mm/s, and rest little of the time, in agreement with previous studies on planarian locomotion (Hagstrom et al., 2015). For simplicity and because these endpoints complemented each other (Supplementary Figure S7), a chemical was classified as a hit if there was a defect in either speed or fraction of time resting.

Considering both endpoints together, 43 chemicals (49%) caused decreased locomotion in at least one worm type (full worms or regenerating tails) and time point. The majority of these chemicals (31 of 43) caused behavioral effects at nonlethal concentrations (Figure 3.7 and Supplementary Table 3). Overall, pesticides comprised the most hits on unstimulated behavior (11 chemicals each for day 7 full and regenerating planarians, and 8 chemicals each for day 12 full and regenerating planarians) (Figure 3.3E-H). In fact, considering the entire library, planarian unstimulated behavior was the most sensitive to the effects of the pesticide rotenone with defects as low as 101nM in full worms at day 7 and in regenerating tails at days 7 and 12. Interestingly, rotenone-exposed day 12 full worms did not display defects in unstimulated behavior, suggesting potential transient toxicity or adaptation over time. Loss or gain of hits between day 7 and day 12 were found with several other chemicals (Figure 3.4A). Moreover, although the majority of chemicals affected both full worms and regenerating tails, some effects were worm type-specific (Figure 3.4B). Together, these demonstrate the power of assaying

toxicity at multiple endpoints and developmental stages to discern the temporal dynamics of toxicity.

In addition to hits which caused decreased activity (due to decreased speed and/or increased time resting), in 8 instances we observed one or two chemical concentrations with induced hyperactivity (due to increased speed and/or decreased time resting compared to controls) (Supplementary Table 4). In fact, the pesticide heptachlor caused hyper-activity in lower concentrations but hypo-activity in higher concentrations in day 12 regenerating tails (Figure 3.3C).

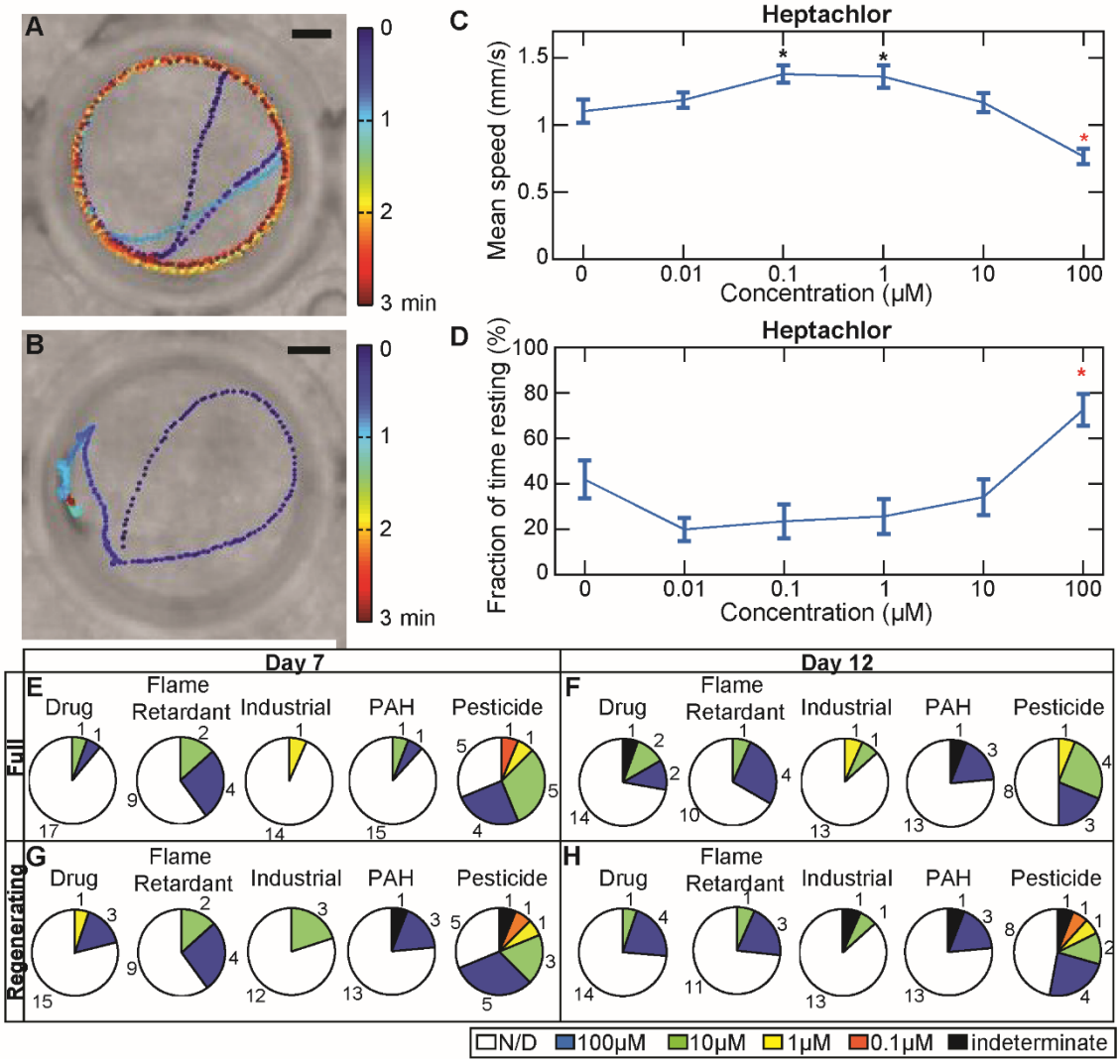


Figure 3.3. Unstimulated behavior: gliding and resting. (A) Representative center of mass (COM) track of one gliding planarian color-coded by time. (B) Representative color-coded COM track of a planarian which started to rest after approximately 1 minute. Scale bars: 2mm. (C-D) Example of dose-response curves of (C) mean speed and (D) mean fraction of time spent resting with standard error as error bars, for same groups of regenerating tails in Heptachlor at Day 12. Stars indicate significant differences from controls ($p<0.05$), showing either hyper-(black, increased locomotor activity) or hypo-activity (red, decreased locomotor activity). (E-F) Distributions of chemicals with defects in unstimulated behavior and their LOEL by chemical class for full worms (E-F, top row) and regenerating tails (G-H, bottom row) at day 7 (left) or day 12 (right). Chemicals which were not found to have an effect on unstimulated behaviors at the tested concentrations are marked as N/D for "not determined". Chemicals with non-monotonic dose-response curves are marked as "indeterminate".

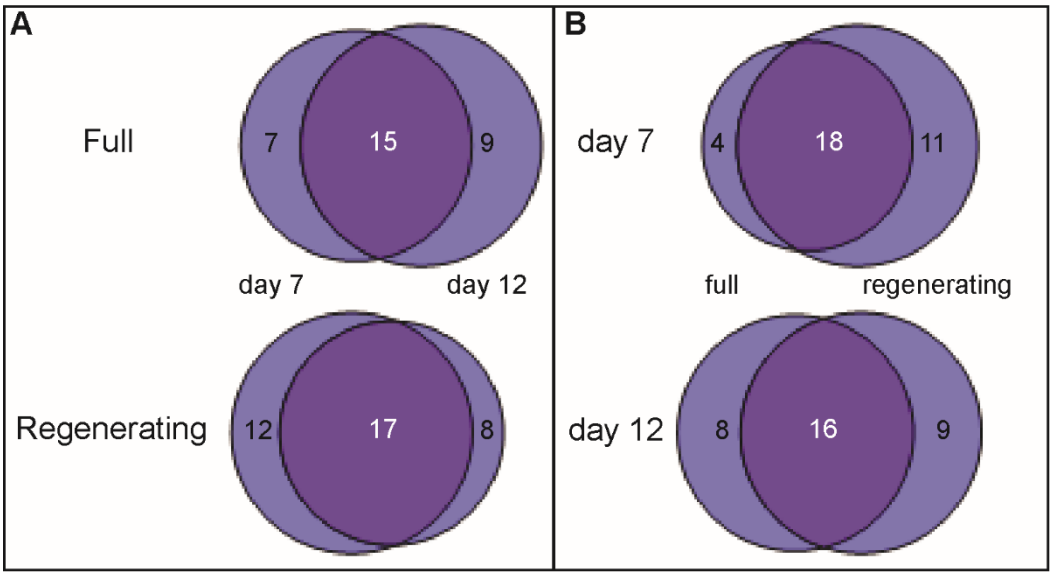


Figure 3.4. Comparison of time-points and worm types for unstimulated behavior hits. (A) Considering both unstimulated behavioral endpoints together, comparison of hits that were conserved between day 7 and day 12 in either full worms (top) or regenerating tails (bottom). (B) Considering both unstimulated behavioral endpoints together, comparison of hits that were conserved between full worms and regenerating tails at either day 7 (top) or day 12 (bottom). All comparisons are performed per chemical, irrespective of concentration.

Stimulated behaviors: phototaxis, thermotaxis and scrunching

Planarians are known to be sensitive to a variety of environmental stimuli, including light and low and high temperatures (Birkholz and Beane, 2017; Cochet-Escartin et al., 2015; Inoue et al., 2004; Inoue et al., 2014; Lambrus et al., 2015; Paskin et al., 2014). For some of these stimuli, it has been shown that different neuronal subpopulations are involved in the animal's characteristic responses to the stimuli (Currie and Pearson, 2013; Inoue et al., 2014; Nishimura et al., 2010). We, therefore, assayed three different stimulated behaviors (phototaxis, thermotaxis and scrunching; Figure 3.5) to potentially differentiate between specific and general neurotoxicity.

First, we tested the planarians response to light (phototaxis). Planarians demonstrate negative phototaxis to blue light while being insensitive to red light (Paskin et al., 2014). Inspired by zebrafish photomotor response assays (Kokel and Peterson, 2011; Truong et al., 2014), we exposed planarians to bright light and compared behavior before (background activity) and after the light stimulus (Figure 3.5A). We then scored the number of planarians which demonstrated phototaxis. We found 15 chemicals induced phototaxis defects in at least one worm type (full or regenerating planarian) and one time point (day 7 or 12), making this the least sensitive of the tested endpoints. However, the majority of these chemicals (9) caused effects at nonlethal concentrations (Supplementary Table 5). The most hits were found in day 7 regenerating tails. Day 7 regenerating hits were found to largely overlap with hits in eye regeneration and unstimulated behavior (Figure 3.6A), suggesting these animals have significant regeneration delays. This is exemplified by the chemical Bis(tributyltin)oxide, which showed the most potent effects on planarian phototaxis, with a LOEL of 0.5μM in both worm types and time points. At this concentration, regenerating tails also had defects in eye regeneration, unstimulated

behavior (day 7 and 12) and scrunching, in the absence of lethality, suggesting a strong defect in regeneration. Similar defects were also found in full animals, but in the presence of lethality. The majority of hits at either day were not shared between full animals and regenerating tails (Supplementary Figure S8B).

We also evaluated how the chemicals affected the planarians' ability to react to a temperature gradient (thermotaxis, Figure 3.5B). The gradient was established using a custom peltier setup to induce individual temperature gradients in each well, thus incorporating our previous manual screening setup (Hagstrom et al., 2015) into the automated screening of 48-well plates. 16 (~18%) of the tested chemicals demonstrated defects in thermotaxis. These active chemicals were mostly evenly distributed among the chemical classes, consisting of 5 industrial chemicals, 4 drugs, 3 flame retardants, 3 pesticides and 1 PAH. In addition, we observed that adults and regenerating animals were often affected differently, with some chemicals only affecting one worm type and not the other, with regenerating tails generally showing greater sensitivity (Supplementary Figure S8C). Moreover, the majority of these effects (10 of the 16 chemicals, ~63%) showed specific neurotoxic effects at nonlethal concentrations (Figure 3.7 and Supplementary Table 6) suggesting that this is a sensitive endpoint to discern sublethal neurotoxicity, particularly in developing animals. Planarian thermotaxis was most sensitive to the drug Tetraethylthiuram disulfide and the pesticide Aldicarb with LOELs of ~10μM for regenerating tails and full worms, respectively. However, at the same concentration, Aldicarb also caused hypoactivity in the unstimulated behavior assay, suggesting the thermotaxis defect may be a consequence of decreased locomotion. Tetraethylthiuram disulfide, on the other hand, caused thermotaxis defects in the absence of locomotion defects, suggesting defects in thermoreception.

Lastly, we evaluated the planarians' ability to react to noxious stimuli. Scrunching is a musculature-driven escape gait in planarians, characterized by asymmetric elongation-contraction cycles (Cochet-Escartin et al., 2015) (Figure 3.5C). This gait can be induced by a variety of noxious stimuli, such as heat, amputation and pH. In our screening platform, scrunching is induced by heating the aquatic temperature of the wells by placing the screening plate on a peltier plate. 38 (~44%) of the tested chemicals caused planarians to be unable to scrunch properly. Similar to lethality, active chemicals in this endpoint were dominated by pesticides (12 chemicals) and flame retardants (10 chemicals). Interestingly, we observed this endpoint to often be affected differentially in the full and regenerating animals, with a slight bias towards regenerating tail pieces, as 14 (37%) chemicals showed increased sensitivity in the regenerating tails and 9 (24%) showed increased sensitivity in the full worms, with 15 toxicants (39%) affecting both worm types at the same concentrations (Supplementary Figure S8D). Among the 38 chemicals that caused scrunching defects, 29 (~76%) showed a scrunching defect with a scrunching LOEL lower than the respective lethality LOEL, for at least one worm type (Figure 3.7 and Supplementary Table 7), suggesting that scrunching is a sensitive endpoint for sublethal neurotoxicity. For example, the most sensitive scrunching defect was seen with the industrial chemical 1-ethyl-3-methylimidazolium diethylphosphate with a LOEL of 101 nM for regenerating tails. This chemical was not found to be lethal to planarians up to the maximum concentration tested (101 μM).

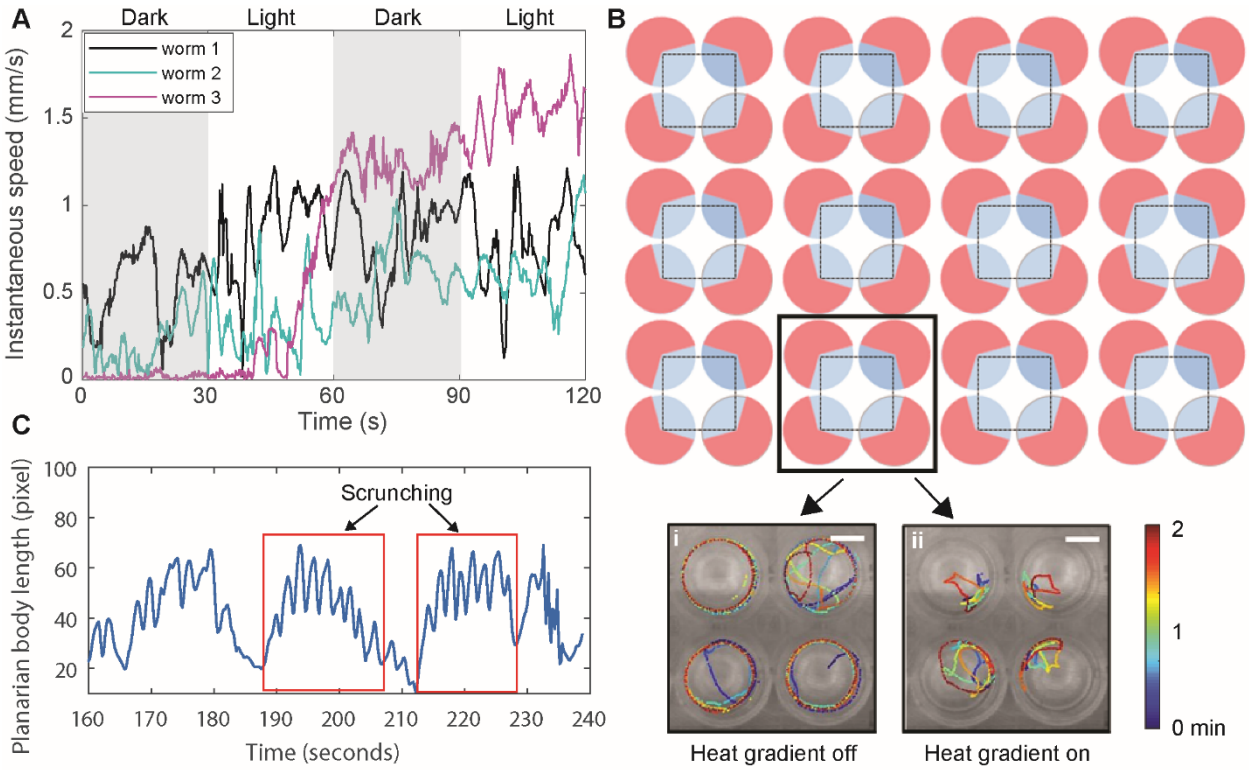


Figure 3.5. Stimulated behaviors. (A) Planarians exhibiting phototaxis respond to alternating light and dark cycles with increasing speed. Examples of 3 full worms in DMSO controls at day 7 were plotted. (B) Schematic of thermotaxis. 12 peltier elements (squares) were evenly distributed to create a heat gradient across each well. The cold area (blue sectors) in each well was defined as the area of a sector of 120° in the analysis. Insets show tracks, color-coded by time, of representative planarian responses to the heat gradient. Both images show the motion of 4 planarians in 4 wells over 2 minutes with either the heat gradient (i) off or (ii) on. Scale bar: 5mm. (C) Representative plot of planarian body length over a short time period (160-240 seconds) in the scrunching assay. The body length oscillations which fulfilled the scrunching criteria in the plot are in a red box. The observed low-frequency oscillations are mostly the worm's turns and head wiggling.

Because the tested endpoints are not necessarily independent from each other, we evaluated the extent of agreement between endpoints that may be correlated. For example, phototaxis and thermotaxis responses rely on animal locomotion to respond to the respective stimuli. Moreover, defects in eye regeneration could be expected to be correlated with defects in phototaxis. We don't, however, expect all hits to be concordant, since the blue light, which was used in the phototaxis assay, can be sensed by photoreceptors in the eyes and pigment in the body epithelium (Birkholz and Beane, 2017). While the majority of phototaxis hits in the regenerating tails were also hits in eye regeneration and/or unstimulated behavior (Figure 3.6A), 1 hit was found in phototaxis alone, suggesting that this assay does add additional sensitivity beyond the other endpoints. Similarly, in full worms, 2 hits were found which were not hits in the unstimulated behavior assay (Supplementary Figure S8A). Moreover, in both thermotaxis and scrunching (Figure 3.6B-C), a large proportion of hits were found to overlap with unstimulated behavior hits, though endpoint-specific effects were found in all cases. Together, these comparisons demonstrate the value of the large repertoire of planarian behaviors to be able to discern subtler neurotoxic effects from general systemic toxicity or gross motor defects.

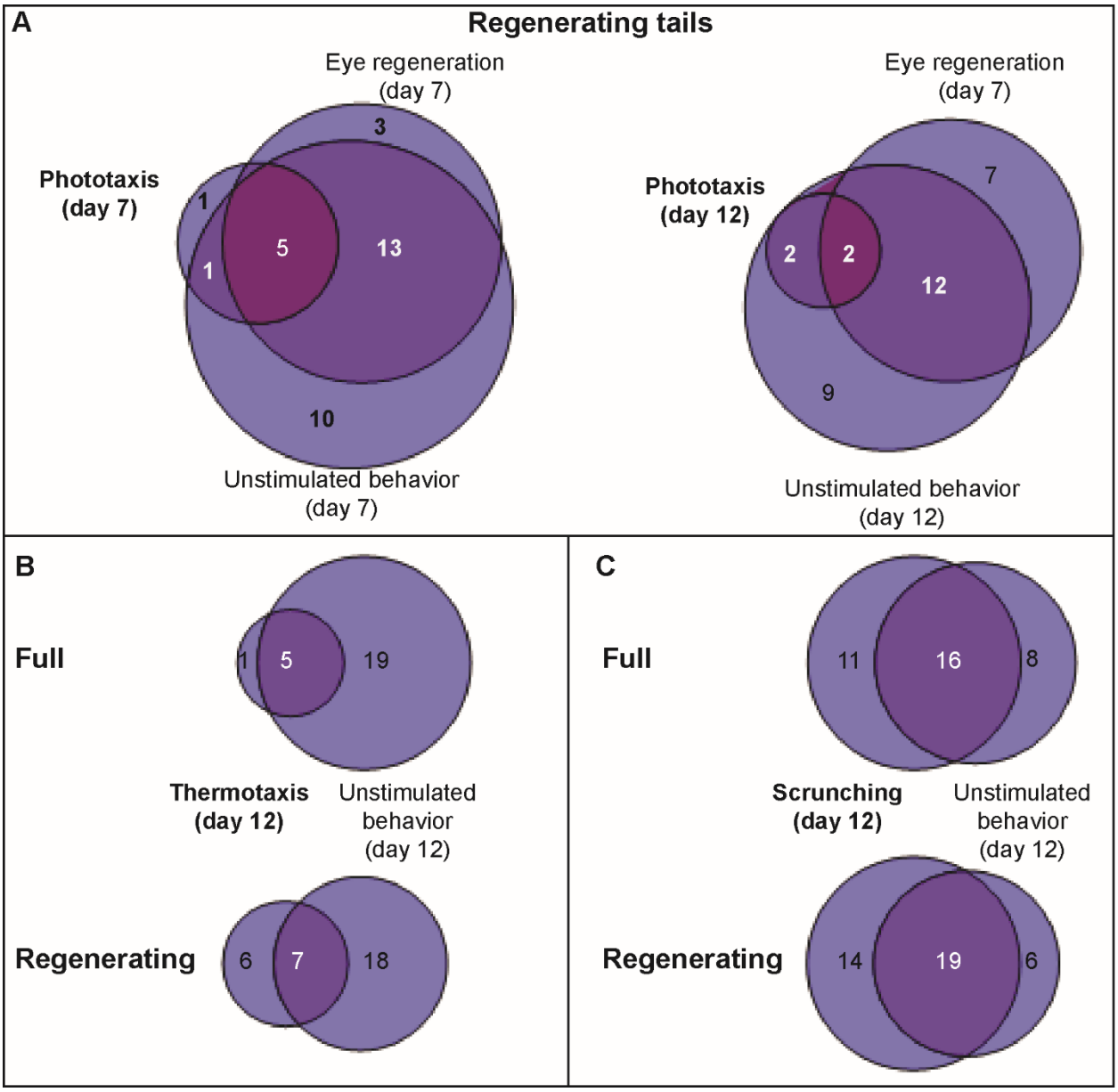


Figure 3.6. Comparison of shared hits in stimulated vs unstimulated behaviors. (A) Venn diagram of overlap of hits in day 7 eye regeneration, with day 7 (left) or day 12 (right) phototaxis and unstimulated behavior assays in regenerating tails. (B) Venn diagram of hits in thermotaxis and unstimulated behavior at day 12 for full worms (top) and regenerating tails (bottom). (C) Venn diagram of hits in scrunching and unstimulated behavior at day 12 for full worms (top) and regenerating tails (bottom).

Sensitivity of endpoints and global response

Through the discussion of the individual assays, we have shown that the different endpoints possess different sensitivities to different toxicities of the tested chemical compounds. Figure 3.7 provides a visual summary of these findings in the case of the regenerating tails (see Supplementary Figure S9 for full worms), allowing for direct comparison of the endpoint sensitivities and selectivity. Furthermore, we applied Ward's method of clustering to summarize the hits of all active compounds (49) for regenerating tails (Figure 3.8) and full worms (47 chemicals) across all endpoints (Figure 3.9), similar to (Truong et al., 2014). Endpoints were clustered into 3 major groups: lethality/morphology endpoints, unstimulated behavior/scrunching and phototaxis/thermotaxis, suggesting endpoints in the same cluster might be functionally related. Some of these clusters seem to represent particular toxic signatures for the different chemical classes (Table 3.3). For example, the majority of pesticides were active in the lethality, unstimulated behavior and scrunching assays. Interestingly, while full worms exposed to pesticides showed more hits (higher class concordance) in lethality, the regenerating tails had more hits in scrunching, suggesting differential effects on the adult and developing nervous system. There was also concordance of endpoints in full worms exposed to flame retardants, with most of the flame retardants being hits in lethality and scrunching. These were also the most concordant endpoints for the regenerating tails exposed to flame retardants, but with slightly less concordance. No obvious signatures were found for any of the other chemical classes, which also generally showed less activity across all planarian endpoints.

When comparing active versus inactive compounds, we found that 41 of the active chemicals are shared hits between full planarians and regenerating tails. When comparing potency, we found 13 chemicals were developmentally selective with lower overall LOELs in

regenerating tails than that in full worms (Table 3.2). Our ability to directly compare the effect of chemicals on the brain of adult (full/intact) and developing (regenerating) animals is a unique strength of the planarian system.

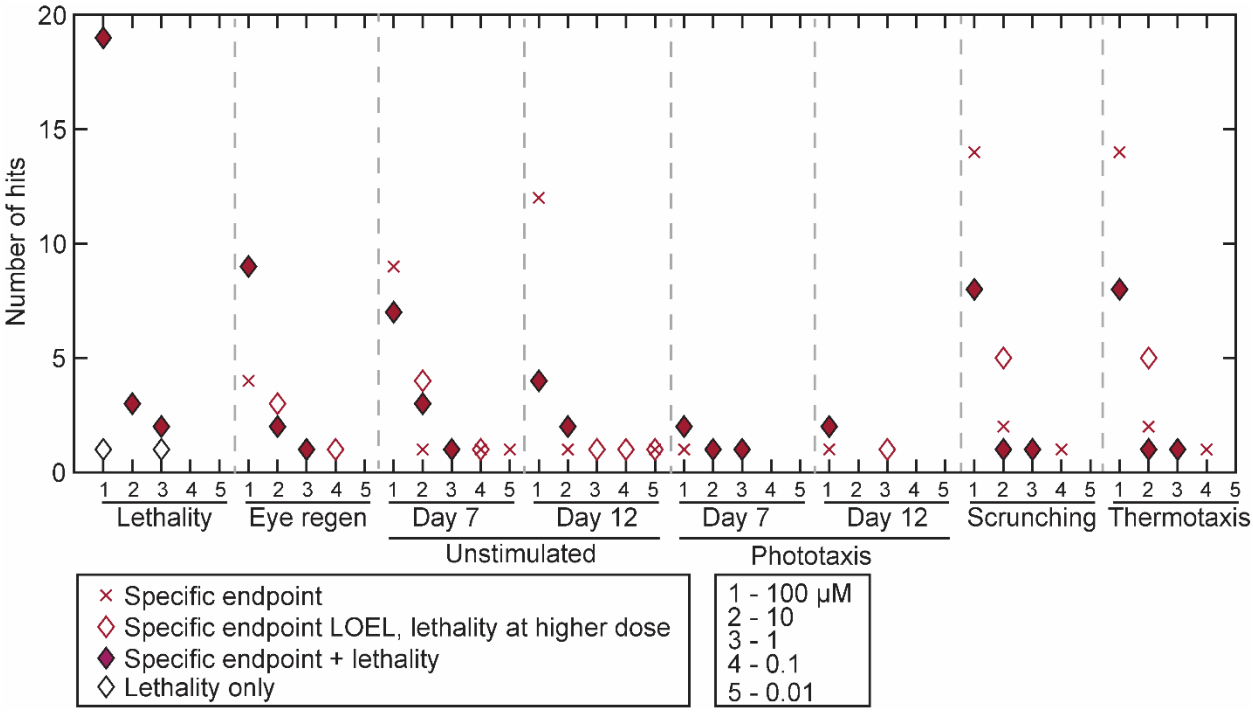


Figure 3.7. Analysis of LOEL by endpoint. Regenerating tail LOELs for each endpoint, separated into 5 concentration classes, listed highest to lowest (1-5). Most chemicals were tested in the range of 0.01-100µM (see legend). However, BDE-153, Chryene and Dibenz(a,h)anthracene were tested at 0.005-50µM, Bis(tributyltin) oxide at 0.5-5000nM, Benzo[g,h,i]perylene at 0.4-4000 nM, and 2,3,7,8-Tetrachlorodibenzo-p-dioxin at 0.04 – 400 nM, due to low solubility in DMSO. Each endpoint LOEL is categorized and counted (y-axis) based on the co-occurrence of lethality at the same or higher concentrations.

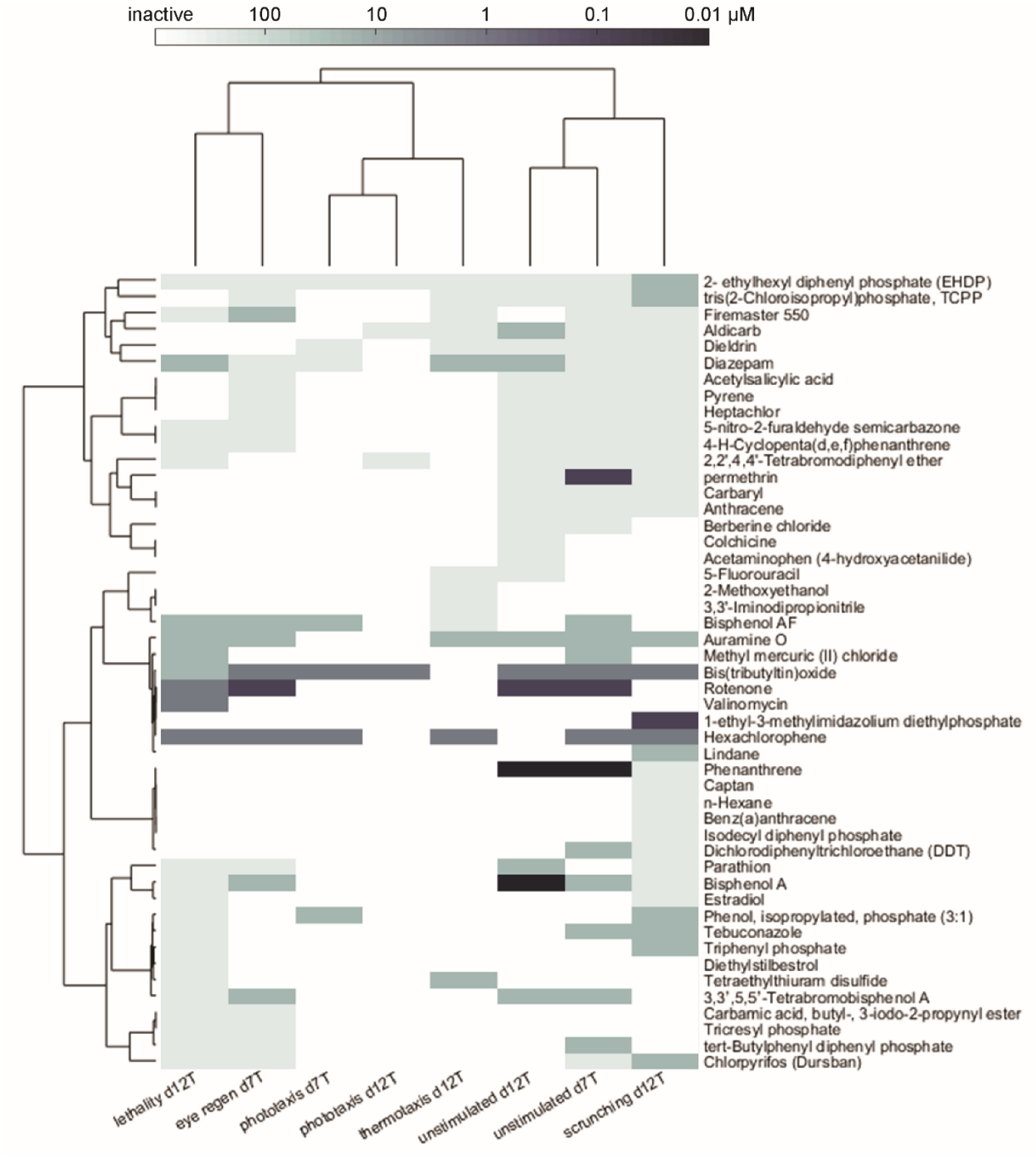


Figure 3.8. Summary of screening results for regenerating tail. Bicluster heat map of chemicals affecting at least one endpoint in regenerating tails with LOEL color-coded. The hits were clustered using Ward's method by calculating Euclidean distance between LOELs.

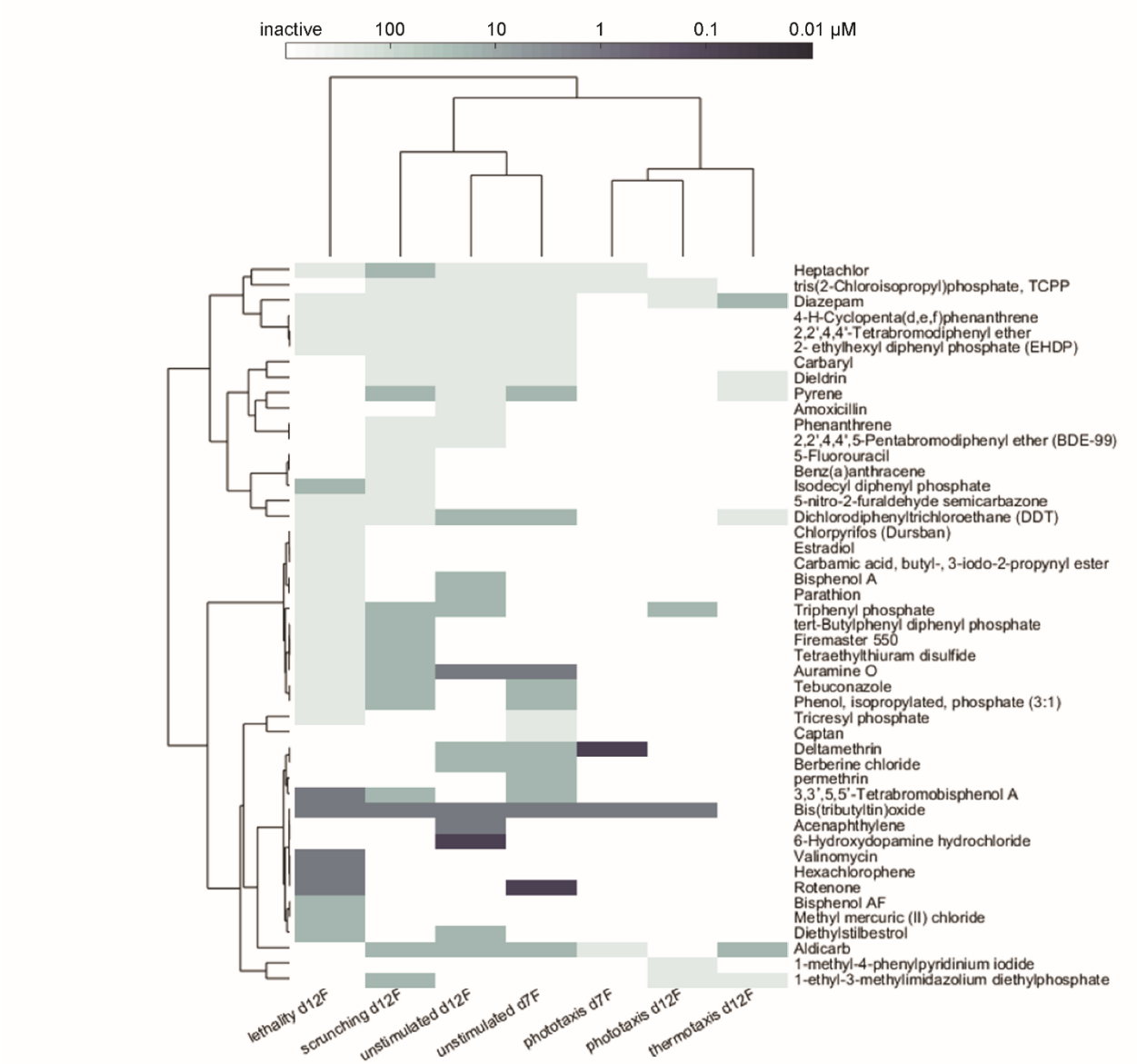


Figure 3.9. Summary of screening results in full planarians. Bicluster heat map of chemicals affecting at least one endpoint in full planarians with LOEL color-coded. The hits were clustered using Ward's method by calculating Euclidean distance between LOELs.

Table 3.2. Developmentally selective chemicals. Chemicals which had overall lower LOELs in regenerating tails than in full planarians.

Class	Chemical	Selective endpoints
Drug	Colchicine	Unstimulated day 12
Industrial	1-ethyl-3-methylimidazolium diethylphosphate	Scrunching
	2-Methoxyethanol	Thermotaxis
	3,3'-Iminodipropionitrile	Thermotaxis
	Bisphenol A	Unstimulated day 12*
	n-Hexane	Scrunching
PAH	Anthracene	Unstimulated day 7/12, Scrunching
	Phenanthrene	Unstimulated day 7* /12*
Pesticide	Chlorpyrifos (Dursban)	Scrunching
	Lindane	Scrunching
	Permethrin	Unstimulated day 7*
Negative	Acetaminophen	Unstimulated day 12
	Acetylsalicylic acid	Unstimulated day 7/12, Scrunching

* dose was non-monotonic

Table 3.3. Summary of percentage of actives observed in different toxicant classes in all endpoints for either full worms (F) or regenerating tails (R). Percentages are based on the total number of chemicals in the respective class. The values were color coded.

Endpoints	Day	Drug (19)		Flame retardant (15)		Industrial(15)		PAH(17)		Pesticide (16)		Negative(5)	
		F	R	F	R	F	R	F	R	F	R	F	R
Lethality	12	37%	37%	60%	53%	20%	20%	6%	6%	56%	44%	0%	0%
Eye	7	NA	16%	NA	40%	NA	20%	NA	12%	NA	38%	NA	20%
Unstimulated	7	11%	21%	40%	40%	7%	20%	12%	24%	69%	69%	0%	20%
	12	26%	26%	33%	27%	13%	13%	24%	24%	50%	50%	0%	0%
Phototaxis	7	0%	11%	7%	13%	0%	7%	0%	0%	25%	13%	0%	0%
	12	11%	0%	13%	13%	7%	0%	0%	0%	25%	13%	0%	0%
Thermotaxis	12	5%	21%	0%	20%	7%	27%	6%	0%	19%	13%	0%	0%
Scrunching	12	21%	21%	67%	47%	13%	27%	24%	29%	44%	75%	0%	20%

Discussion

Robustness of screen and best practices

Robustness and reliability of screening are major concerns in the evaluation and verification of toxicology models (Judson et al., 2013). One aspect is reproducibility of results between independent experimental runs (technical replicates). Therefore, in our screen, we assayed each chemical concentration in 3 independent runs and provide the data for direct comparison of the replicates in Supplementary File 1. The majority of hits were reproducible with significant activity in all 3 runs, with on average 73% shared for all runs for all endpoints with full and regenerating planarians (Supplementary Table 8). However, variability among runs was evident in some cases potentially due to technical artifacts and variability among animal populations, as described below.

First, technical issues in the scrunching assay contributed to the observed spread in the data for this endpoint. Specifically, in 3.8% of the screened plates (N=522 plates), the contact between the plate and the peltier used for administering the noxious heat stimulus was inadequate, causing variability in the scrunching response. However, the same dose-dependent trends seen in the replicates with properly functioning peltier contact was still evident in these malfunctioning replicates (Supplementary Figure S11).

Next, to account for possible effects of well position within a single plate, we rotated the position of the different chemical concentrations among runs by shifting each concentration down 2 rows with each replicate. This revealed the existence of an “edge effect”, whereby planarians located at the outermost rows of the plate displayed a relatively higher lethality rate when compared to the planarians located in the plate interior at the same concentration (Supplementary Figure S10). We thus conclude, as others have previously (Truong et al., 2014),

that alteration of well position for a given chemical concentration between replicates is an important aspect of ensuring reliability of results and thus enhancing screen robustness.

Finally, the planarian's diet turned out to be a significant source of biological variability affecting planarian fitness and behavior. Varying quality of food batches caused a measurable influence on the animals' sensitivity to chemical exposure (see Supplementary Information Section 3 for details) and calls for standardization of food quality to eliminate this source of variability within and between experiments and labs.

To minimize the effects of inter-run variability arising from any of these factors, we excluded hits that were determined through a single run and did not have consistent effects across the triplicates (see Material and Methods and Supplementary Figure S5).

Negative controls

The NTP 87-compound library contained 5 compounds indicated as negative controls (acetaminophen, acetylsalicylic acid, D-glucitol, L-ascorbic acid and saccharin sodium salt hydrate). All negative controls were inactive in full planarians. In contrast, in regenerating tails, while 3 of the 5 negative controls (D-glucitol, L-ascorbic acid and saccharin) showed no effects, at least one endpoint was affected by acetaminophen and acetylsalicylic acid. Acetaminophen caused decreased unstimulated speed in day 12 regenerating tails at the highest concentration tested (103μM). However, this hit was right at the biological relevance cutoff (see Materials and Methods), so it is possible that it is a false positive or potentially mild effect.

Acetylsalicylic acid caused defects in eye regeneration, unstimulated behavior (day 7 and 12) and scrunching in regenerating tails (but not full worms) at the highest tested concentration (99.5μM) suggesting developmental defects. While these chemicals were selected by the NTP to

be inactive controls at the tested concentrations, toxicity has been observed with these compounds previously. Data collected by the NTP from different public databases shows that acetaminophen and acetylsalicylic acid have been reported to have "other" and developmental/other toxicity, respectively (https://sandbox.ntp.niehs.nih.gov/neurotox/). Moreover, these 2 compounds have been associated with toxicity in multi-generation and developmental mammalian guideline studies, respectively, reported on ToxRefDB (Hagstrom et al.) For example, oral exposure of 1% (1.43 mg/kg body weight) acetaminophen to Swiss CD-1 mice for 14 weeks caused multi-generational effects on reproduction and growth (Reel et al., 1992), while single dose oral exposure to 500 mg/kg acetylsalicylic acid caused teratogenesis in rats (DePass and Weaver, 1982). Thus, these findings point toward a potential toxic effect of these compounds on developmental processes in various animal systems.

Comparison of hits with existing planarian toxicology data

For some of the chemicals tested in this screen, previous largely manual toxicology studies on planarians exist. We therefore compared our results with the published literature to evaluate concordance (Table 3.4). Of note, while we studied chronic exposure in both full and regenerating planarians, most of the previous studies evaluated either only regeneration and/or acute exposure. Direct comparisons between different experiments are difficult to make because of differences in experimental methods (chemical concentrations tested, exposure conditions and duration, worm type (full/regenerating), data and statistical analysis, number of replicates, etc.), and differences in planarian species used, which may have differing sensitivity. Together, this experimental heterogeneity emphasizes the need for uniform testing guidelines going forward.

The zebrafish community faces similar challenges, for example see (Truong et al., 2014), with different labs using different experimental methodologies.

Table 3.4. Comparison of results with previous planarian studies. As necessary, concentrations were converted to µM for ease of comparison.

	This screen		Previously published				
Chemical	**LOEL (µM)**	**Endpoints affected**	**Species**	**LOEL (µM)**	**Endpoints affected**	**Exposure duration**	**Reference**
6-Hydroxydopamine hydrochloride	0.1*	Unstimulated behavior (d12F)	*Dugesia gonocephala*	~120†	Mobility	7 days	(Caronti et al., 1999)
Acetaminophen	100	Unstimulated behavior (d12R)	*D. japonica*	1000§	Lethality	4 days	(Li, 2013a)
Acetylsalicylic acid	100	Eye regeneration, unstimulated behavior (d7R, d12R), scrunching (d12R)	*D. japonica*	520§	Lethality	4 days	(Li, 2013a)
Acrylamide	N/D	none	*D. japonica*	100	Gliding speed, brain regeneration	8,15 days	(Hagstrom et al., 2015)¶
Benz(a)anthracene	100	Scrunching (d12F/R)	*D. dorotocephala*	Not available	Lethality	3 months	(Best and Morita, 1982)
Berberine chloride	10	Unstimulated behavior (d7F/R, d12F/R)	*D. japonica*	50	Eye regeneration, mobility	7 days	(Balestrini et al., 2014)
Bisphenol A	0.01*	Lethality (F/R), eye regeneration, unstimulated behavior (d7R, d12F/R), scrunching (d12R)	*D. japonica*	~20§ 2	Lethality Regeneration	4 days 7 days	(Li, 2013b) (Li, 2014)
Carbaryl	100	Unstimulated behavior (d7F/R, day12F/R), scrunching (day12F/R)	*Dugesia tigrina*	1	Mobility	2h	(Feldhaus et al., 1998)
Chlorpyrifos	10	Lethality (F/R), eye regeneration, unstimulated behavior (d7R), scrunching (d12R)	*D. japonica; D. dorotocephala*	1 5	Lethality, unstimulated behavior, brain regeneration, thermotaxis; lethality, acute behavior, head regeneration	8,15 days 7 days	(Hagstrom et al., 2015)¶ (Villar et al., 1993)
Colchicine	100	Unstimulated behavior (d12F)	*D. dorotocephala*	200	Regeneration	10 days	(McWhinnie, 1955)

Table 3.4. Comparison of results with previous planarian studies. As necessary, concentrations were converted to µM for ease of comparison (continued).

	This screen		Previously published				
Chemical	**LOEL (µM)**	**Endpoints affected**	**Species**	**LOEL (µM)**	**Endpoints affected**	**Exposure duration**	**Reference**
Dichlorodiphenyltrichloroethane (DDT)	10	Lethality (F), unstimulated behavior (d7F,d12F/T), scrunching (d12F/T), thermotaxis (d12T)	*Polycelis feline, Creobia alpina*	1	Regeneration, mobility	14 days	(Kouyoumjian and Villeneuve, 1979)
Diethylstilbestrol	10	Lethality (F/R), unstimulated behavior (d12F)	*D. japonica*	2§ 2	Lethality Regeneration	4 days 7 days	(Li, 2013b) (Li, 2014)
Estradiol	100	Lethality (F/R), scrunching (d12R)	*D. japonica*	6§ 9	Lethality Regeneration	4 days 7 days	(Li, 2013b) (Li, 2014)
Methyl mercuric (II) chloride	10	Lethality (F/R), Unstimulatd behavior (d7F)	*D. dorotocephala; Polycelis tenuis and Dugesia lugubris*	0.3	Lethality, morphology, regeneration, behavior	5h-10 days	(Best et al., 1981)
Parathion	10	Lethality (F/R), eye regeneration, unstimulated behavior (d12F/T), scrunching (d12T)	*Schmidtea mediterranea*	50	Lethality, regeneration, mobility	1 – 4 days, 7 days, 12 days	(Poirier et al., 2017)
Permethrin	0.1*	Unstimulated behavior (d7F/R, d12R), scrunching (d12R)	*D. japonica*	20	Lethality, gliding speed, eye regeneration, brain regeneration	8,15 days	(Hagstrom et al., 2015)¶
Rotenone	0.1	Lethality (F/R), eye regeneration, unstimulated behavior (d7F/R, d12R)	*D. japonica*	0.01	Lethality, acute behavior	4 days	(Kitamura et al., 2003)
Thalidomide	N/D	none	*D. tigrina*	232	Eye regeneration	5 day exposure, assayed at day 7	(Best and Morita, 1982)

*dose response was non-monotonic. †only 1 tested concentration. §LC_{50}. ¶see Supplementary Information Section 6 for more details

Strengths and current limitations of the planarian as a model for developmental neurotoxicity

The performance of this 87-compound screen revealed both the strengths and weaknesses of the planarian screening platform, as summarized in Table 3.5. As with any toxicology system, the planarian system has its limitations. However, when appropriately utilized, this system can add value to the existing testing pipeline through its unique strengths, such as the ability to screen adults and developing animals in parallel with the same assays to delineate developmental-specific effects and differentiate between DNT and general neurotoxicity (Table 3.2). For example, of the 38 known developmental neurotoxicants in this library (Supplementary Table 1, (Ryan et al., 2016)), 10 (1 drug, 5 industrial, and 4 pesticides) had greater effects in regenerating planarians, with lower overall LOELs than full planarians.

Table 3.5. Summary of the strengths and weaknesses of the planarian toxicology system.

Strengths	Weaknesses
• Cost- and time-effective screen within 12 days compared to months in mammalian systems • Invertebrate system • Amenable to full automation • Easy administration of compounds in the water • Many different behavioral readouts, some with known cellular/molecular pathways • Ability to study adult and developing animals in parallel with the same assays • Allows for multi-generational studies	• Limited morphological endpoints due to simple anatomy • May be missing some relevant toxicological targets • Potential water solubility issues and loss of toxicants into the environment • Unknown PK/PD parameters (e.g. internal concentrations and xenobiotic metabolism); • Single route of exposure (absorption) • Clonal animals, no genetic diversity

Another strength of the planarian system is the large repertoire of quantitative behavioral readouts that allow coverage of a wide spectrum of neuronal functions that are currently not assayed in other medium-throughput animal systems, such as zebrafish larvae. Moreover, the molecular mediators of some of these behaviors have been characterized (Birkholz and Beane, 2017; Inoue et al., 2014; Nishimura et al., 2010), allowing for insight into mechanisms of neurotoxicity. For example, 10 μM Tetraethylthiuram disulfide was found to selectively disrupt thermotaxis in regenerating tails, but not full planarians, in the absence of other affected endpoints. Planarian thermotaxis has been shown to be mediated by Transient Receptor Potential (TRP) channels (Inoue et al., 2014), and Tetraethylthiuram disulfide has been found to be a selective agonist for human TRPA1 *in vitro* (Maher et al., 2008). Additionally, regenerating planarians were found to be highly sensitive to rotenone, a pesticide and mitochondrial disruptor. We observed significant defects in full and regenerating tails unstimulated behavior and eye regeneration at concentrations as low as 101nM. In rodent models, the effects of rotenone on retinal neurodegeneration and locomotor activity have been well documented (Alam et al., 2004; Normando et al., 2016; Rojas et al., 2008). The similarity of these affected endpoints in both models suggests that similar molecular pathways are targeted in the same way. Together, these demonstrate the utility of the range of planarian morphological and behavioral endpoints to connect adverse functional outcomes with mechanisms, which are likely conserved in higher organisms, including mammals and humans.

In the NTP 87-compound library, 38 chemicals were denoted as known developmental neurotoxicants (Supplementary Table 1) from previous *in vivo* and *in vitro* studies (Ryan et al., 2016) and 23 (~61%) were active in planarian regenerating tails. Concordance varied by class from most to least: pesticide (13/14), industrial (4/10), and drug (6/14). No PAHs or flame

retardants were listed as known developmental neurotoxicants. Moreover, in our companion paper (Hagstrom et al.), we found that of the 28 chemicals in this library with associated quality mammalian guideline studies available on the U.S. EPA Toxicity Reference Database, 20 (71%) were active in regenerating planarians. Some of these false negatives may be due to absence of the relevant biological targets in planarians. For example, the inactivity of thalidomide, an infamous teratogen with suggested effects on angiogenesis (Stephens et al., 2000), in planarians may not be surprising given their lack of a circulatory system.

Other factors need to be taken into account when evaluating concordance, such as the extent of uptake and bioavailability in the animals. The reported concentrations in this study are nominal water concentrations and the internal concentrations within the planarians are unknown. Thus, it is uncertain whether inactivity is due to loss of chemical to the plastic, lack of absorption into the planarian, insufficient metabolic machinery, or other pharmacokinetic (PK) differences. For example, since chemical uptake in planarians occurs through the skin or pharynx (Balestrini et al., 2014; Kapu and Schaeffer, 1991) and planarians possess a protective mucus coating (Martin, 1978; Pedersen, 2008), certain chemical classes may be unable to effectively penetrate into the animal. Future research will have to determine the PK and pharmacodynamics (PD) of this system, and identify which compounds are bioavailable, to be able to connect activity with the relevant exposure in mammals and humans. While this study focused on the planarian system, a companion study in this special issue (Hagstrom et al.) performs a direct comparison using this NTP 87-compound library between the planarian and zebrafish systems, and available mammalian data. Together, both studies demonstrate the added value of comparative screening in multiple complementary models to assay a larger swath of chemical and biological space.

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Chapter 3, in full, is a reformatted reprint of the material as it appears in Toxicological Sciences 2018 (Zhang, Siqi; Hagstrom, Danielle; Hayes, Patrick; Graham, Aaron; and Collins, Eva-Maria S. "Multi-behavioral endpoint testing of an 87-chemical compound library in freshwater planarians," Toxicological Sciences 2018). The version of record is available online at: https://academic.oup.com/toxsci/advance-article/doi/10.1093/toxsci/kfy145/5034903. Use of this manuscript in the dissertation herein is covered by the rights permitted to the authors by Oxford Journals. The dissertation author was the primary author in this paper. Siqi Zhang, Danielle Hagstrom and Eva-Maria S. Collins designed the experiments. Siqi Zhang designed and built the automatic screening platform, designed and performed the screening experiments and analyzed and interpreted the majority of the data. Danielle Hagstrom set up the chemicals and animals, and analyzed and interpreted part of the data. Patrick Hayes and Aaron Graham developed the neural network algorithm to analyze eye regeneration data. We thank Alex Fields for help setting up the rail system used in the screening platform, Jessica Soong for help manufacturing the thermotaxis peltier holder and accessories of Fresnel lens design, Noopur Khachane for help setting up the power control system and the temperature measurement kit, Yingtian He for help with animal care and data analysis and Jared Estrada for help with animal care. We also thank Robert Tanguay and Lisa Truong for discussion. Siqi Zhang and Danielle Hagstrom were the primary investigators and authors of this material.

References

Alam, M., Mayerhofer, A. and Schmidt, W. J. (2004). The neurobehavioral changes induced by bilateral rotenone lesion in medial forebrain bundle of rats are reversed by L-DOPA. *Behav. Brain Res.* 151, 117–124.

Balestrini, L., Isolani, M. E., Pietra, D., Borghini, A., Bianucci, A. M., Deri, P. and Batistoni, R. (2014). Berberine exposure triggers developmental effects on planarian regeneration. *Sci. Rep.* 4, 4914.

Behl, M., Hsieh, J.-H., Shafer, T. J., Mundy, W. R., Rice, J. R., Boyd, W. A., Freedman, J. H., Hunter, E. S., Jarema, K. A., Padilla, S. and Tice, R.R. (2015). Use of alternative assays to identify and prioritize organophosphorus flame retardants for potential developmental and neurotoxicity. *Neurotoxicol. Teratol.* 52, 181–193.

Behl, M., Ryan, K., Hsieh, J.-H., Parham, F., Shapiro, A., Collins, B. J., Birnbaum, L. S., Bucher, J. R., Walker, N. J., Foster, P. M., et al. (2018). Screening for developmental neurotoxicity (DNT) at the National Toxicology Program: The future is now! *Toxicol. Sci.*

Birkholz, T. R. and Beane, W. S. (2017). The planarian TRPA1 homolog mediates extraocular behavioral responses to near-ultraviolet light. *J. Exp. Biol.* 220, 2616–2625.

Boyd, W., Smith, M. and Freedman, J. (2012). Caenorhabditis elegans as a model in developmental toxicology. In *Developmental Toxicology* (ed. Harris, C.) and Hansen, J. M.), pp. 15–24. Humana Press.

Boyd, W. A., Smith, M. V., Co, C. A., Pirone, J. R., Rice, J. R., Shockley, K. R. and Freedman, J. H. (2015). Developmental effects of the ToxCast^{TM} phase I and phase II chemicals in Caenorhabditis elegans and corresponding responses in zebrafish, rats, and rabbits. *Environ. Health Perspect.* 124, 586–93.

Bronsted, H. V. (1955). Planarian regeneration. *Biol. Rev.* 30, 65–126.

Buchanan, J. W. (1935). An analysis of physiological states responsible for antero-posterior disintegration in Planaria dorotocephala. *Protoplasma* 22, 497–512.

Buttarelli, F. R., Pellicano, C. and Pontieri, F. E. (2008). Neuropharmacology and behavior in planarians: Translations to mammals. *Comp. Biochem. Physiol. - C Toxicol. Pharmacol.* 147, 399–408.

Carter, J. A., Lind, C. H., Truong, M. P. and Collins, E.-M. S. (2015). To each his own. *J. Stat. Phys.* 161, 250–272.

Cebrià, F. (2007). Regenerating the central nervous system: how easy for planarians! *Dev. Genes Evol.* 217, 733–48.

Cochet-Escartin, O., Mickolajczk, K. J. and Collins, E.-M. S. (2015). Scrunching: a novel escape gait in planarians. *Phys. Biol.* 12, 55001.

Collins, F. S., Gray, G. M. and Bucher, J. R. (2008). Transforming environmental health protection. *Science* 319, 906–7.

Currie, K. W. and Pearson, B. J. (2013). Transcription factors lhx1/5-1 and pitx are required for the maintenance and regeneration of serotonergic neurons in planarians. *Development* 140, 3577–88.

DePass, L. R. and Weaver, E. V. (1982). Comparison of teratogenic effects of aspirin and hydroxyurea in the Fischer 344 and Wistar strains. *J. Toxicol. Environ. Health* 10, 297–305.

Dunkel, J., Talbot, J. and Schötz, E.-M. (2011). Memory and obesity affect the population dynamics of asexual freshwater planarians. *Phys. Biol.* 8, 26003.

Faul, F., Erdfelder, E., Lang, A.-G. and Buchner, A. (2007). G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behav. Res. Methods* 39, 175–191.

Hagstrom, D., Truong, L., Zhang, S., Tanguay, R. L. and Collins, E.-M. S. (2018) Comparative analysis of zebrafish and planarian model systems for developmental neurotoxicity screens using an 87-compound library Title. *Toxicol. Sci.*

Hagstrom, D., Cochet-Escartin, O., Zhang, S., Khuu, C. and Collins, E.-M. S. (2015). Freshwater planarians as an alternative animal model for neurotoxicology. *Toxicol. Sci.* 147, 270–285.

Hagstrom, D., Cochet-Escartin, O. and Collins, E.-M. S. (2016). Planarian brain regeneration as a model system for developmental neurotoxicology. *Regeneration* 3, 65–77.

Hill, A. J., Teraoka, H., Heideman, W. and Peterson, R. E. (2005). Zebrafish as a model vertebrate for investigating chemical toxicity. *Toxicol. Sci.* 86, 6–19.

Hori, I. and Kishida, Y. (1998). A fine structural study of regeneration after fission in the planarian Dugesia japonica. *Hydrobiologia* 383, 131–136.

Inoue, T., Kumamoto, H., Okamoto, K., Umesono, Y., Sakai, M., Alvarado, A. S. and Agata, K. (2004). Morphological and functional recovery of the planarian photosensing system during head regeneration. *Zoolog. Sci.* 21, 275–283.

Inoue, T., Yamashita, T. and Agata, K. (2014). Thermosensory signaling by TRPM is processed by brain serotonergic neurons to produce planarian thermotaxis. *J. Neurosci.* 34, 15701–14.

Judson, R., Kavlock, R., Martin, M., Reif, D., Houck, K., Knudsen, T., Richard, A., Tice, R. R., Whelan, M., Xia, M. and Huang, R. (2013). Perspectives on validation of high-throughput assays supporting 21st century toxicity testing. *ALTEX* 30, 51–56.

Kapu, M. M. and Schaeffer, D. J. (1991). Planarians in toxicology. Responses of asexual Dugesia dorotocephala to selected metals. *Bull. Environ. Contam. Toxicol.* 47, 302–7.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn.* 203, 253–310.

Kokel, D. and Peterson, R. T. (2011). Using the zebrafish photomotor response for psychotropic drug screening. *Methods Cell Biol.* 105, 517–524.

Lambrus, B. G., Cochet-Escartin, O., Gao, J., Newmark, P. A., Collins, E.-M. S. and Collins, J. J. (2015). Tryptophan hydroxylase is required for eye melanogenesis in the planarian Schmidtea mediterranea. *PLoS One* 10, e0127074.

Maher, M., Ao, H., Banke, T., Nasser, N., Wu, N.-T., Breitenbucher, J. G., Chaplan, S. R. and Wickenden, A. D. (2008). Activation of TRPA1 by farnesyl thiosalicylic acid. *Mol. Pharmacol.* 73, 1225–1234.

Martin, G. G. (1978). A new function of rhabdites: Mucus production for ciliary gliding. *Zoomorphologie* 91, 235–248.

Mineta, K., Nakazawa, M., Cebria, F., Ikeo, K., Agata, K. and Gojobori, T. (2003). Origin and evolutionary process of the CNS elucidated by comparative genomics analysis of planarian ESTs. *Proc. Natl. Acad. Sci. U. S. A.* 100, 7666–71.

Morita, M. and Best, J. B. (1984). Effects of photoperiods and melatonin on planarian asexual reproduction. *J. Exp. Zool.* 231, 273–282.

Nishimura, K., Kitamura, Y., Taniguchi, T. and Agata, K. (2010). Analysis of motor function modulated by cholinergic neurons in planarian Dugesia japonica. *Neuroscience* 168, 18–30.

Normando, E. M., Davis, B. M., De Groef, L., Nizari, S., Turner, L. A., Ravindran, N., Pahlitzsch, M., Brenton, J., Malaguarnera, G., Guo, L. and Somavarapu, S. (2016). The retina as an early biomarker of neurodegeneration in a rotenone-induced model of Parkinson's disease: Evidence for a neuroprotective effect of rosiglitazone in the eye and brain. *Acta Neuropathol. Commun.* 4, 86.

Pan, S. J. and Yang, Q. (2010). A survey on transfer learning. *IEEE Trans. Knowl. Data Eng.* 22, 1345–1359.

Paskin, T. R., Jellies, J., Bacher, J. and Beane, W. S. (2014). Planarian phototactic assay reveals differential behavioral responses based on wavelength. *PLoS One* 9, e114708.

Pedersen, K. J. (2008). Sliime-secreting cells of planarians. *Ann. N. Y. Acad. Sci.* 106, 424–443.

Reel, J. R., Lawton, A. D. and Lamb, J. C. (1992). Reproductive toxicity evaluation of acetaminophen in Swiss CD-1 mice using a continuous breeding protocol. *Fundam. Appl. Toxicol.* 18, 233–239.

Rink, J. C. (2013). Stem cell systems and regeneration in planaria. *Dev. Genes Evol.* 223, 67–84.

Rojas, J. C., Saavedra, J. A. and Gonzalez-Lima, F. (2008). Neuroprotective effects of memantine in a mouse model of retinal degeneration induced by rotenone. *Brain Res.* 1215, 208–217.

Ross, K. G., Currie, K. W., Pearson, B. J. and Zayas, R. M. (2017). Nervous system development and regeneration in freshwater planarians. *Wiley Interdiscip. Rev. Dev. Biol.* 6, e266.

Ryan, K. R., Sirenko, O., Parham, F., Hsieh, J. H., Cromwell, E. F., Tice, R. R. and Behl, M. (2016). Neurite outgrowth in human induced pluripotent stem cell-derived neurons as a high-throughput screen for developmental neurotoxicity or neurotoxicity. *Neurotoxicology* 53, 271–281.

Scholz, S. (2013). Zebrafish embryos as an alternative model for screening of drug-induced organ toxicity. *Arch. Toxicol.* 87, 767–769.

Stephens, T. D., Bunde, C. J. and Fillmore, B. J. (2000). Mechanism of action in thalidomide teratogenesis. *Biochem. Pharmacol.* 59, 1489–99.

Tejeda-Benitez, L. and Olivero-Verbel, J. (2016). Caenorhabditis elegans, a biological model for research in toxicology. In *Reviews of Environmental Contamination and Toxicology* (ed. de Voogt, W.), pp. 1–35. Springer, Cham.

Truong, L., Reif, D. M., St Mary, L., Geier, M. C., Truong, H. D. and Tanguay, R. L. (2014). Multidimensional in vivo hazard assessment using zebrafish. *Toxicol. Sci.* 137, 212–33.

Tsuji, R. and Crofton, K. M. (2012). Developmental neurotoxicity guideline study: Issues with methodology, evaluation and regulation*. *Congenit. Anom. (Kyoto).* 52, 122–128.

Umesono, Y., Tasaki, J., Nishimura, K., Inoue, T. and Agata, K. (2011). Regeneration in an evolutionarily primitive brain--the planarian Dugesia japonica model. *Eur. J. Neurosci.* 34, 863–9.

Yang, X., Kaj, K. J., Schwab, D. J. and Collins, E.-M. S. (2017). Coordination of size-control, reproduction and generational memory in freshwater planarians. *Phys. Biol.* 14, 36003.

Zhang, G., Truong, L., Tanguay, R. L. and Reif, D. M. (2017). A new statistical approach to characterize chemical-elicited behavioral effects in high-throughput studies using Zebrafish. *PLoS One* 12, e0169408.

Chapter 4: Comparative analysis of zebrafish and planarian model systems for developmental neurotoxicity screens using an 87-compound library

Chapter 4, in full, is a reformatted reprint of the material as it appears Toxicological Sciences 2018 (Hagstrom, Danielle; Truong, Lisa; Zhang, Siqi; Tanguay, Robert L; and Collins, Eva-Maria S. "Comparative analysis of zebrafish and planarian model systems for developmental neurotoxicity screens using an 87-compound library", Toxicological Sciences 2018).

The supplementary data is available online (https://academic.oup.com/toxsci/advance-article-abstract/doi/10.1093/toxsci/kfy180/5053695).

Abstract

There is a clear need to establish and validate new methodologies to more quickly and efficiently screen chemicals for potential toxic effects, particularly on development. The emergence of alternative animal systems for rapid toxicology screens presents valuable opportunities to evaluate how systems complement each other. In this article, we compare a chemical library of 87-compounds in two such systems, developing zebrafish and freshwater planarians, by screening for developmental neurotoxic effects. We show that the systems' toxicological profiles are complementary to each other, with zebrafish yielding more detailed morphological endpoints and planarians more behavioral endpoints. Overall, zebrafish was more sensitive to this chemical library, yielding 86/87 hits, compared to 50/87 hits in planarians. The difference in sensitivity could not be attributed to molecular weight, Log K_{ow} or the bioconcentration factor. Of the 87 chemicals, 28 had previously been evaluated in mammalian developmental neuro- (DNT), neuro- or developmental toxicity studies. Of the 28, 20 were hits in the planarian, and 27 were hits in zebrafish. Eighteen of the 28 had previously been identified as DNT hits in mammals and were highly associated with activity in zebrafish and planarian behavioral assays in this study. Only 1 chemical (out of 28) was a false negative in both zebrafish and planarian systems. Differences in endpoint coverage and system sensitivity illustrate the value of a dual systems approach to rapidly query a large chemical-bioactivity space and provide weight-of-evidence for prioritization of chemicals for further testing.

Introduction

There is increasing recognition in the field of toxicology that the high expense, low throughput, and uncertainty of traditional rodent testing are inadequate to evaluate the ever-growing number of environmental chemicals. Alternative integrated systems, such as zebrafish, nematodes, and planarians, have emerged to fill these gaps because they are small, easy to breed and maintain, comparatively inexpensive, and develop quickly (Boyd et al., 2012; Boyd et al., 2015; Hagstrom et al., 2016; Hill et al., 2005; Tejeda-Benitez and Olivero-Verbel, 2016; Truong et al., 2014). Use of these models, with their own unique strengths and limitations, is an effective bridge between *in vitro* and whole animal/mammalian testing methods, allowing for rapid exploration of adverse outcome pathways. Comparative screening in multiple such animal models covers more chemical-biological space, thus providing greater weight of evidence for prioritization of chemical hazards.

Recently, the NIEHS National Toxicology Program (NTP) initiated a collaboration wherein different labs studying a variety of alternative models, including *in vitro* cell culture systems, zebrafish, and planarians, screened a library of 87 unique compounds, allowing for cross-system comparisons (Behl et al., 2018). This library consisted of known and suspected developmental neurotoxicants and 5 designated negative control chemicals, expanded from a previously tested library of 80 compounds (Ryan et al., 2016). Here we compare the results of screening this library in a developmental zebrafish model and freshwater planarian system.

Zebrafish embryos and larvae have become a popular animal model for developmental (Linney et al., 2004) and neurotoxicity studies (Bailey et al., 2013; He et al., 2014) because this system strikes an optimal balance among model complexity and tractability. This system is amenable to high-throughput whole animal screening (Geier et al., 2018; Noyes et al., 2015;

Truong et al., 2014) and maintains strong developmental and physiological similarity to higher vertebrates. In particular, the external development and optical transparency of zebrafish embryos allows for detailed morphological studies. Moreover, 84% of genes that have been associated with a human disease have an obvious orthologue in zebrafish (Howe et al., 2013).

The asexual freshwater planarian *Dugesia japonica* has recently emerged as a high-throughput, alternative whole animal model for developmental neurotoxicology (Hagstrom et al., 2015; Hagstrom et al., 2016). Planarians have a high capacity to regenerate, which allows them to reproduce asexually by binary fission (Malinowski et al., 2017) and fully regenerate a centralized nervous system (Cebrià, 2007; Rink, 2013). In this asexual species, neurodevelopment is achieved through neuroregeneration and can be induced by decapitation. Because of the similar sizes of adult and regenerating/developing animals, the planarian system offers the unique ability to screen both life stages in parallel with the same assays to identify potential effects specific to development. Moreover, planarians possess a large repertoire of quantifiable behaviors, many of which can be assayed in a fully automated fashion, including thermotaxis, phototaxis, and a characteristic escape response, providing distinct readouts of neuronal function (Cochet-Escartin et al., 2015; Hagstrom et al., 2016; Inoue et al., 2014; Paskin et al., 2014; Zhang et al., 2018).

Because the systems differ in their developmental timeline, we compared the chemical effects at two different stages: 1) early development (24 hours post fertilization (hpf) in the zebrafish and day 7 in the planarian), when the animals begin to form major anatomical structures, and 2) late development (day 5 or 120 hpf in the zebrafish and day 12 in the planarian), when the animals have essentially completed development. We assayed for developmental delays, morphological abnormality, mortality and altered behavioral endpoints.

Eighty-six out of 87 chemicals were bioactive in the zebrafish, while 50 were bioactive in regenerating planarians. Physicochemical properties (e.g. molecular weight, log K_{ow} and bioconcentration factor (BCF)) did not account for bioactivity differences between the models. Of the 87 chemicals, 28 were previously associated with high quality mammalian toxicity data in ToxRefDB. The regenerating planarian responses to 20 of these 28 chemicals (~71%) and the zebrafish responses to 27 of the 28 (96%) were concordant with mammalian outcomes. By utilizing both model systems, the field might rapidly query more chemical-bioactivity space for guided prioritization of testing in mammals using mechanistic insight gained from the breadth of morphological and behavioral endpoints jointly provided by the two systems.

Material and methods

Chemical library: Supplementary Table 1 lists the chemicals composing the NTP 87-compound library and their purity, consisting of 5 chemical classes (drugs, industrial chemicals, flame retardants, polycyclic aromatic hydrocarbons (PAHs), and pesticides) as well as 5 intended negative control chemicals. More details on the composition of the library can be found in Behl et al, the introduction of this special issue (Behl et al., 2018). Chemicals were provided as ~20 mM stocks, dissolved in dimethyl sulfoxide (DMSO). Four duplicate chemicals were provided in the 91-compound library tested in the zebrafish study but were not provided in the planarian library.

Planarian studies: Asexual *D. japonica* were used for the planarian screen. Details of the experimental procedures and data analysis, as well as the analyzed data for each endpoint can be found in (Zhang et al., 2018). In brief, 3 replicates of n=8 (n=24 in total) developing/regenerating and adult/intact planarians were each screened for every chemical of the library, generally at 10 nM, 100 nM, 1 μM, 10 μM and 100 μM. Exposure began on day 1, after the amputation of regenerating planarians, and the planarians were kept in sealed 48-well plates (Genesee Scientific, San Diego, CA) for 12 days, with screening occurring on days 7 and 12 (Figure 4.1). The DMSO levels were kept constant at 0.5% and n=8 control populations exposed to the 0.5% DMSO solvent control were included in every plate. We have previously shown this DMSO concentration has no effect on planarian morphology or behaviors (Hagstrom et al., 2015). All raw data associated with the screen are available on the Dryad Digital Repository (doi: 10.5061/dryad.mk6m608).

Zebrafish studies: Tropical 5D wildtype adult zebrafish were housed at Oregon State University, Sinnhuber Aquatic Research Laboratory (SARL) in standard 14h light/10h dark-light

cycle. Embryos were collected, cleaned and staged prior to dechorionation at 4 hpf. The chorions were enzymatically removed and at 6 hpf, one embryo was placed per well in round bottom 96-well plates prefilled will 100 µL of embryo media. The chemicals were digitally dispensed directly from the 20 mM stocks into the test wells using a Hewlett Packard D300e, and all wells normalized to 0.64% DMSO (vol/vol). Up to 1% DMSO has no adverse effects to developing zebrafish (Maes et al., 2012). Each chemical was tested at 0, 1, 2, 4.5, 9, 18, 34, and 67 µM and sealed with parafilm to minimize evaporation and shaken gently overnight at 235 rpm (Truong et al., 2016). Ten chemicals (2,3,7,8-Tetrachlorodibenzo-p-dioxin, Bis(tributyltin)oxide, Benzo[g,h,i]perylene, hexachlorophene, rotenone, tetraethylthiuram disulfide, deltamethrin, methyl mercury (II) chloride, saccharin sodium salt hydrate, and valinomycin) were retested at lower concentrations as the first assessment resulted in 100% mortality or morbidity in all concentrations. A list of the concentrations can be found in Supplementary Table 2. Developmental toxicity was assessed by evaluating mortality, developmental progression, spontaneous movement, and notochord distortion at 24 hpf. Behavioral assessments were conducted at 24 hpf (Embryo photomotor response, EPR) using the Photo-motor Response Assessment Tool (Reif et al., 2016) and at 120 hpf using Viewpoint LifeScience Zebraboxes (Truong et al., 2014; Zhang et al., 2017). At 120 hpf, additional developmental and morphological toxicity endpoints were assessed including: mortality, yolk sac edema, curved or bent body axis in either direction, missing or smaller/larger than normal eye, shortened or malformed snout, malformed jaw, malformed or missing otic vesicle, pericardial edema, malformation or necrosis of the brain, malformed, missing or disorganized somites, malformed or missing pectoral fin, malformed or missing caudal fin, lack of pigment or over pigmentation, lack of circulation, truncated body, failure of swim bladder to inflate, bent notochord and/or tail,

and response to touch. After chemical exposure, embryos were not exposed to visible light until administration of the EPR test at 24 hpf (Reif et al., 2016). The test consisted of 30 s of darkness (IR light, Background); first 1 s pulse of intense VIS light, 9 s darkness (Excitation); second pulse of VIS light, 10 s darkness (Refractory) (Truong et al., 2014). Statistical significance was calculated for each interval using a Kolmogorov-Smirnov test (K-S) with a threshold of $p < 0.01$. The 120 hpf larval photomotor response assay (LPR) was conducted just prior to morphological evaluation. In the LPR, larvae experience a total of 4 light cycles, each cycle consisting of 3 min of alternating light and dark. Wells with mortality or malformed animals were excluded from the subsequent analysis. An entropy score was calculated for each interval and compared to the control to compute a relative ratio, as described in (Zhang et al., 2017). Statistical significance was determined using a K-S test ($p < 0.01$) and a relative ratio of $> 10\%$ or $< 10\%$. All analyses were conducted using custom R scripts previously described (R Core Team, 2016). All zebrafish morphological concentration response plots are available in Supplementary Materials (Supplementary Figure 2).

Meta-analysis: Supplementary File 1 contains the lowest effect levels (LELs) identified for each endpoint in both the zebrafish and planarian screens. LELs which did not pass an additional criterion of having at least two consecutive concentration hits for an endpoint (statistically significant for zebrafish, and statistically and biologically significant for planarians – see (Zhang et al., 2018) for more details) are marked in red. In parenthesis is the new LEL after applying the criteria (i.e. excluding concentration-independent hits). Incidences of endpoint abnormality (hit detection) in the two systems were compared based on whether the chemical was deemed active or not, irrespective of concentration. Each chemical was scored in MATLAB (Mathworks, Nattick, MA) as either active in planarians, active in zebrafish, active in both, or

inactive. We did not consider the concentration which elicited a response due to differences in the concentrations tested and because of the unknown relationships between the nominal aquatic concentrations and the internal concentration within the animals. As such, we have no available information to determine the actual amount of toxicants inside the animals, making direct comparisons of concentration inappropriate. Therefore, the data was converted to binary data: hit or no hit (Supplementary Table 3). The endpoints were organized into classes (Table 4.1) to compare similar endpoints between the two systems. Furthermore, to compare similar developmental stages, endpoints at 24 hpf in the zebrafish studies were compared with endpoints at day 7 in the planarian studies and 120 hpf (day 5) zebrafish endpoints with day 12 planarian endpoints. Comparisons were also made at the system level, considering any affected endpoint.

Table 4.1. Classes of endpoints used in the two systems.

Endpoint Class	Zebrafish endpoints	Planarian endpoints
Mortality	Mortality (24 hpf, 120 hpf)	Lethality (day 7, day 12)
Morphology	Developmental progress (24 hpf) Spontaneous movement (24 hpf) Notochord distortion (24 hpf) Curved/bent axis (120 hpf) Brain malformation or necrosis (120 hpf) Malformed or missing caudal fin (120 hpf) No circulation or blood flow (120 hpf) Eyes malformed, missing or abnormal sized (120 hpf) Heart malformation, pericardial edema (120 hpf) Malformed jaw (120 hpf) Malformed or missing otic (120 hpf) Malformed or missing pectoral fin (120 hpf) Lack of pigmentation or over pigmentation (120 hpf)	Eye regeneration (day 7)
Behavior	Embryo photomotor response (EPR: 24hpf) Larval photomotor response (LPR: 120hpf)	Unstimulated behavior (day 7 and 12) Phototaxis (day 7 and 12) Scrunching (day 12) Thermotaxis (day 12)

The physicochemical and *in vivo* animal data analysis was completed using custom R scripts (R Core Team, 2016). The chemical-physical property log octanol/water partition coefficient (log K_{ow}) and bioconcentration factor (BCF) was obtained using EPISuite v4.11 (http://www.epa.gov/opptintr/exposure/pubs/episuite.htm). The CAS was input into EPISuite and the experimental log K_{ow} and BCF values were used when available. The molecular weight and CAS were provided by the NTP.

To compare the results of the zebrafish and planarian screens with *in vivo* animal data, the publicly available animal data were downloaded from the US Environmental Protection Agency's Toxicity Reference Database (ToxRefDB) (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data). The August 2014 data release of the study treatment file ("toxrefdb_study_tg_effect_endpoint_AUG2014_FOR_PUBLIC_RELEASE") and the summary file ("toxrefdb_nel_lel_noael_loael_summary_AUG2014_FOR_PUBLIC_RELEASE") were used. The studies used in this analysis were those that fit the usability criteria of "Acceptable guideline (post -1998), "Acceptable Guideline (pre-1998)", and "Acceptable Non-guideline". As the 87 compounds were selected to be potential developmental or developmental neurotoxicants, the study types were filtered to developmental (DEV), multi-generational (MGR), neurotoxicity (NEU) and developmental neurotoxicity (DNT). There was no filter on the species used. One point to consider is that the current version of ToxRefDB only houses chemicals that cause adverse effects in animal studies. The 59 other chemicals found in this 87-compound library could either have been in ToxRefDB but with studies that did not follow guideline protocols (which we deemed not usable for these analyses) or were negatives. For these reasons, the concordance study was benchmarked to the 28 active chemicals. Concordance analysis was

conducted using a custom R script, and the R package, circlize. DEV and MGR study types were mapped to zebrafish and planarian mortality and morphology, while NEU and DNT were mapped to zebrafish EPR and LPR and planarian early and late behavior.

Results

Screening the NTP 87-compound library in both systems

Each of the chemicals of the NTP 87-compound library (Behl et al., 2018; Ryan et al., 2016) were classified as either generally developmentally toxic, developmentally neurotoxic, neurotoxic, or unknown due to limited data (https://sandbox.ntp.niehs.nih.gov/neurotox/). The chemicals were structure and use classified as drug, flame retardant, industrial, PAH, pesticide, or inactive (as defined by the NTP). As Supplemental Figure 1 illustrates, the largest class in the library consisted of drugs (19 of 87; 22%). There were 5 chemicals selected as inactive negative controls by the NTP library curators.

Figure 4.1 shows an overview of the different experimental schemes used for the zebrafish and planarian screens. In both studies, developing animals (either dechorionated 6 hpf zebrafish embryos or amputated planarian tails) were statically exposed to multiple concentrations of each chemical in multi-well plates. For planarian studies, both adult (intact) animals and decapitated animals regenerating a new brain (regenerating tail pieces) were assayed. For ease of comparison to the developing zebrafish, we focused on data associated with the regenerating planarians. The comparison with adult worms can be found in Supplementary Figure 3. Chemical bioactivity was assayed in early development (24 hpf zebrafish and day 7 planarian) and late development (120 hpf zebrafish and day 12 planarian) (Figure 4.1). Dual system screening yielded a significantly larger coverage of endpoints, with zebrafish contributing most of the morphological endpoints (13 vs. 1 in planarians) and the planarian system contributing more behavioral endpoints, covering different behavioral stimuli (light, temperature, noxious heat) and general locomotion compared to photoresponse behaviors, (6 vs. 2 in zebrafish).

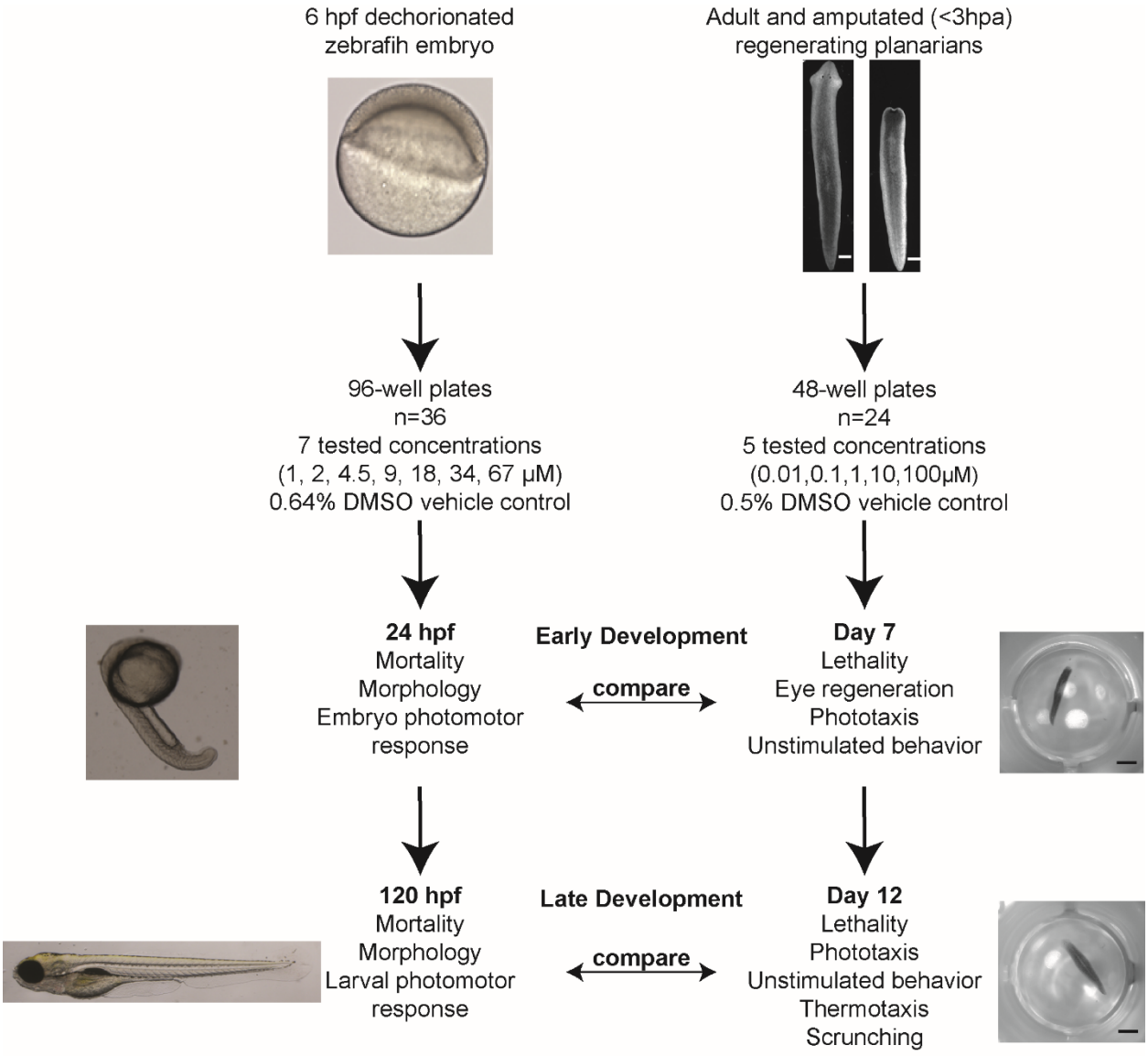

Figure 4.1. Comparison of screening schemes in the zebrafish and planarian systems. Details of the two screens including testing conditions and endpoints by time-point. Table 4.1 summarizes the different morphological endpoints assayed in the zebrafish system. Hpa: hours post-amputation. Scales are as follows: white scale bars: 0.5mm, black scale bars: 2mm; Zebrafish 6 hpf embryo is ~0.7 mm diameter; 24 hpf is1.9 mm long; 120 hpf is 3.9 mm long (https://zfin.org/zf_info/zfbook/stages/index.html).

Concordance of active chemicals between zebrafish and planarians

Considering any assay endpoint, zebrafish were more sensitive indicators of bioactivity, i.e., 86 of 87 (99%) unique chemicals were bioactive (Figure 4.2a). Thirty-two chemicals were hits in morphological endpoints, 49 in the EPR, and 66 in the LPR. Fifteen chemicals were bioactive in all 4 assays. Five chemicals for morphological endpoints, 3 in EPR, and 6 in LPR did not show concentration-dependence (marked in red in Supplementary File 1). Collectively, this did not change our identification of bioactive chemicals, as they were bioactive in the other assays under these new criteria.

Additionally, 50 of the 87 chemicals (57%) were bioactive at any endpoint in regenerating (developing) planarians and 48 (55%) in adult worms (see Supplementary Figure 3). In regenerating planarians, 21 chemicals were hits for eye regeneration (morphology), 31 for at least one early behavior, and 45 for at least one late behavior (Figure 4.2b). Thus, the majority of bioactivity in the planarian system (47 of 50 chemicals, 90%) was detected by behavioral endpoints and almost ¼ (12 of 50) with behavior alone. Six chemicals showed concentration-independent effects (i.e. active at a lower but not higher concentrations) on unstimulated behavior in regenerating planarians (marked in red in Supplementary File 1). However, for 5 of these, effects were still seen at higher concentrations in other endpoints. For one chemical, Chrysene, the only observed effect was concentration-independent hyper-activity in the unstimulated behavior assay. All other hits showed concentration-dependence or only caused effects at the highest concentration tested. Thirteen chemicals were active in all 4 endpoint categories in planarians. All of the developing planarian bioactivity hits were also hits in zebrafish, accounting for 58% of the zebrafish bioactivity hits. The only chemical inactive in both screens was the drug hydroxyurea.

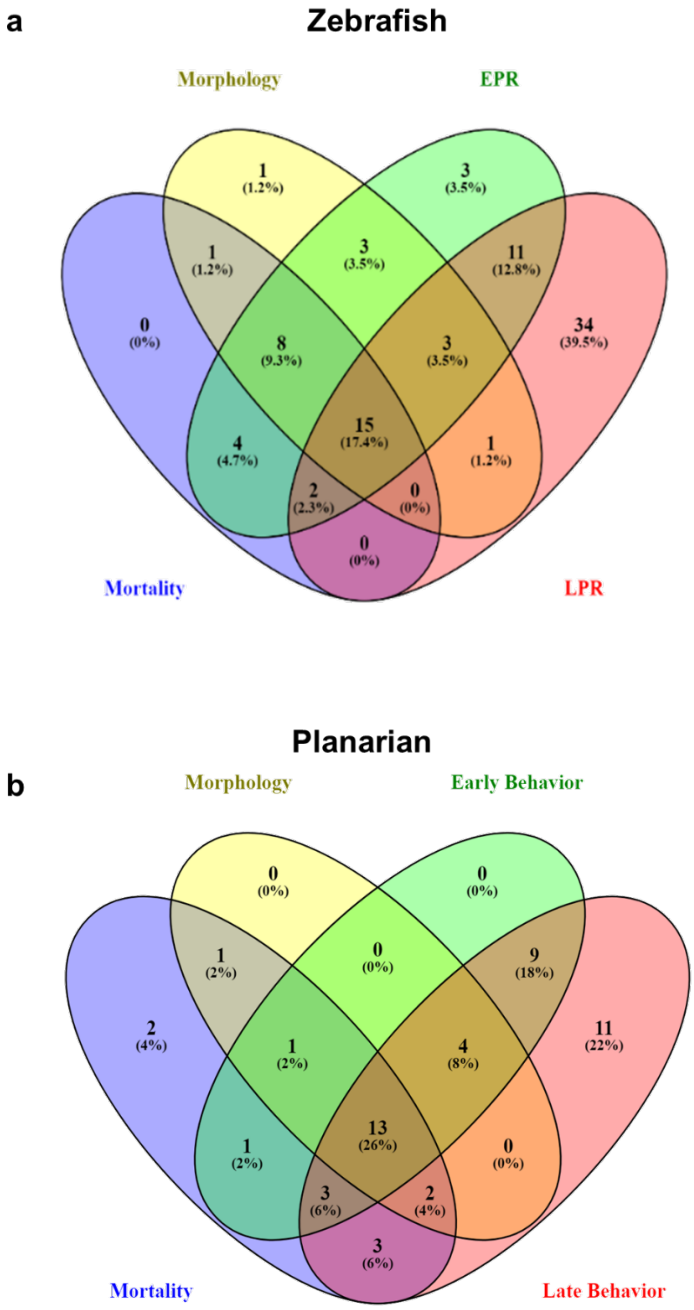

Figure 4.2. **Summary of (a) zebrafish and (b) planarian hits in each endpoint class**. Both model systems were exposed to the 87 chemicals and assessed in 4 assays: morphology, mortality, (a) EPR and LPR in zebrafish, and (b) early and late behavior in planarians.

Similar endpoints - mortality, morphology, behavior – were assessed in each model; thus, we compared the chemical hit rate for each endpoint class (Table 4.1 and Figure 4.3). Because of the similar developmental timelines, early and late endpoints were compared across models and concordance was based on the number of shared hits out of the total in zebrafish. Similar numbers of chemicals were found to be lethal in the two systems, with approximately 70% of these mortality hits being concordant (15/21 and 21/30 for early and late time points, respectively). In addition, 15/32 chemicals were concordant for morphological effects (47%). In both systems, the majority of chemical hits were detected in the behavior endpoints with 25/49 (51%) and 36/66 (55%) chemicals concordant at the early and late time-points, respectively. Similar trends were also found when comparing developing zebrafish to adult planarians, albeit with slightly less concordance, particularly for behavioral endpoints (Supplementary Figure 3).

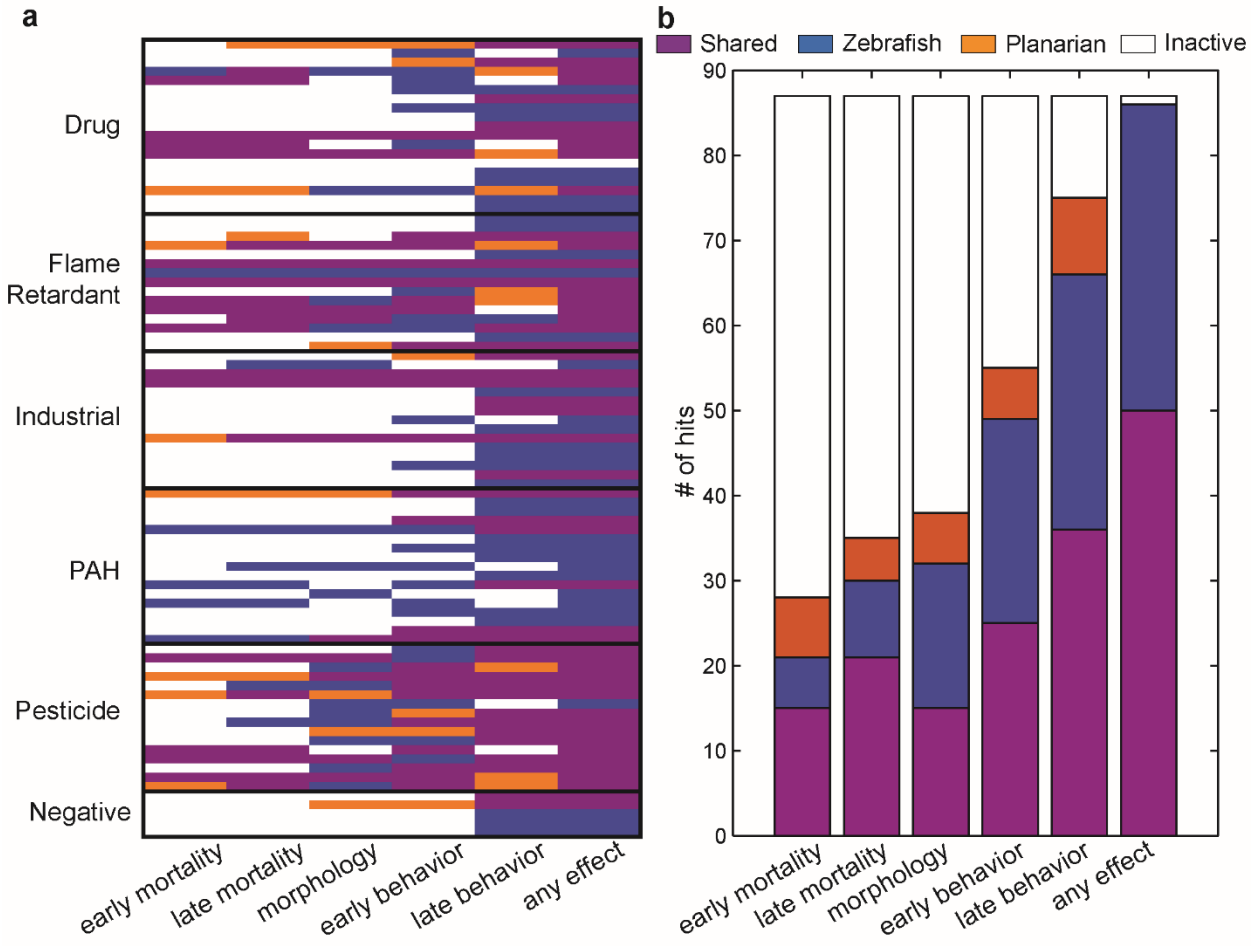

Figure 4.3. Comparison of active hits in the zebrafish and regenerating planarian screens. a) Classification of hits for each chemical (rows), organized by chemical class, whether it was active in both systems (purple), zebrafish only (blue), regenerating planarians only (orange), or inactive (white) in each endpoint class (columns). See Table 4.1 for a description of the endpoints within classes. b) Number of hits in each endpoint classification used in (a).

Comparison to physicochemical properties

The NTP 87-compound library consists of chemicals with a range of physicochemical properties. We focused on properties of putative high relevance to a waterborne exposure paradigm: molecular weight (MW), Log K_{ow} (log of the octanol/water partition coefficient) and BCF (bioconcentration factor). We found that neither MW, Log K_{ow}, nor BCF was entirely predictive of a response for both model systems. The single inactive chemical in the zebrafish screen, hydroxyurea, was not due to high molecular weight (Figure 4.4a), log K_{ow} (Figure 4.4c), or BCF (Figure 4.4d). Similarly, high molecular weight did not explain the instances of negative chemicals in the planarian model (Figure 4.4b) as they were all below 600 g/mol. Log K_{ow} and BCF (Figures 4.4d, f, respectively) were also not readily associated with instances of chemical inactivity. Thus, the overall association of physicochemical parameters with whole animal chemical bioactivity was weak.

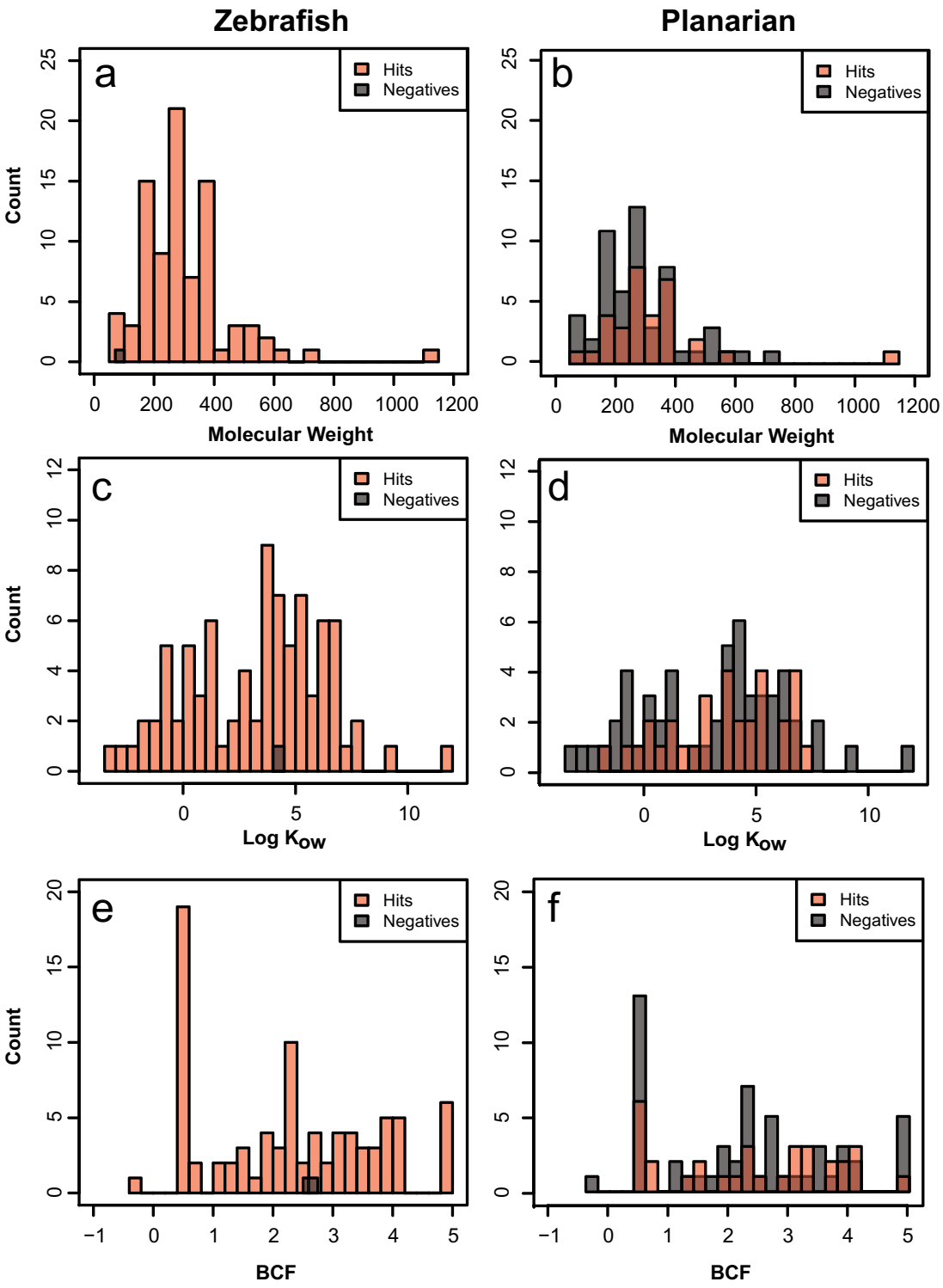

Figure 4.4. **Physicochemical properties of the NTP 87-compound library.** Comparing the (a, b) molecular weight, (c, d) Log K_{ow}, and (e, f) BCF of the inactives and the biological actives in zebrafish (left) and planarian (right).

Concordance with available animal data

The US EPA Toxicity Reference Database (ToxRefDB) houses *in vivo* studies from over 1,000 chemicals and thousands of animal toxicity studies in rat, rabbit, mouse, primate, dog, guinea-pig, hamster, and mink. We found that 28 chemicals in ToxRefDB (Supplementary Table 4) were also common to the NTP 87-compound library. Of note, this shared chemical set mainly consisted of pesticides (12 chemicals), drugs (8 chemicals), and industrial chemicals (5 chemicals) as well as 2 of the designated negative controls and 1 PAH.

By way of dataset comparison, we filtered the 28 ToxRefDB chemicals by adverse response category: 16 were identified in ToxRefDB as developmentally toxic (DEV), 18 as developmentally neurotoxic (DNT), 1 as a neurotoxic (NT) and 15 as multi-generationally toxic (MGR), in their respective studies (Supplementary Table 4).

Among the 28 chemicals common to this study and ToxRefDB, the overall, any effect, hit concordance was 27 of 28 (96%) for zebrafish bioactivity (Figure 4.5a) and 20 of 28 (71%) for regenerating planarian bioactivity (Figure 4.5b). For the 16 chemicals associated with general developmental toxicity in ToxRefDB, using morphology and mortality endpoints, 11 (69%) were hits in zebrafish, 7 (44%) were hits in the regenerating planarian, and 6 (37.5%) were hits in both. Four developmentally toxic chemicals did not show activity in our dual screen in either morphology or mortality: 2-methyloxyethanol, captan, Di(2-ethylhexyl) phthalate, and naphthalene. For the 15 multi-generationally toxic chemicals, 12 (80%) were hits in zebrafish, 6 (40%) were hits in the regenerating planarian, and 6 (40%) were hits in both systems when considering only morphology and mortality. Three of the 15 were inactive: 6-propyl-2-thiouracil, acetaminophen and Di(2-ethylhexyl)phthalate. Eighteen of the 28 shared chemicals had DNT studies, indicating that the 28 chemicals (and the NTP 87-compound library itself) were enriched

with developmental neurotoxicants. For the 18 developmentally neurotoxic chemicals, 17 (95%) were also hits in zebrafish behavior (both EPR and LPR), and 10 (56%) were hits in early and/or late planarian behavior, with all 10 of these being hits in both models. Neither model in the present study detected bioactivity for hydroxyurea. Only one chemical, carbamic acid, had a neurotoxicity study and was a hit in both the zebrafish and planarian. We note that 5 chemicals of the NTP 87-compound library were previously classified as negatives by the library's curators, but 2, acetaminophen and acetylsalicylic acid, were hits in ToxRefDB in MGR and DEV studies, respectively.

For a summary perspective of how the 28 chemicals interacted with the zebrafish and planarian endpoints, we created a chord diagram (Figure 4.5), which links the chemicals to ToxRefDB study types and zebrafish/planarian endpoints. The width of each endpoint or chemical indicates the number of interactions. In the zebrafish chord plot, LPR and morphology had the most interactions and were on par with the ToxRefDB DNT study type (Figure 4.5a). For the planarian, this trend is similar with late behavior being a highly linked endpoint (Figure 4.5b). The chord diagram for both models' endpoint classes (4 each) and the 4 ToxRefDB toxicity types is shown in Figure 4.5c. Both the zebrafish LPR and planarian late behavior endpoints had the most interactions (associated bioactivity with the largest width) with the subset of 28 chemicals, supporting the utility and predictivity of the systems' behavioral endpoints for classifying DNT.

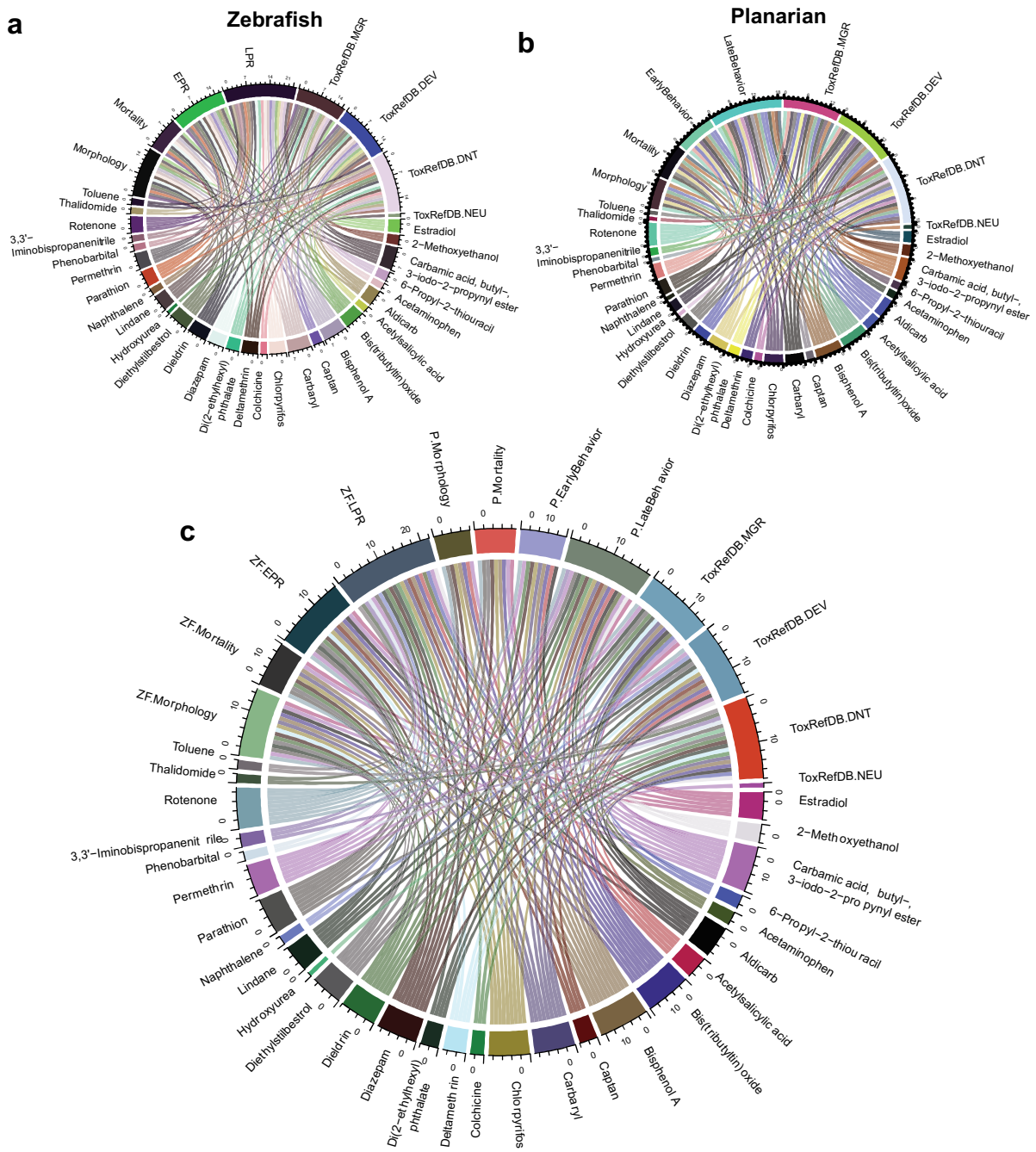

Figure 4.5. Inter-relationship between 28 chemicals, zebrafish and planarian assay endpoints and study types in ToxRefDB. A total of 28 chemicals had *in vivo* animal studies and were linked to **(a)** 4 zebrafish endpoints, **(b)** 4 planarian endpoints and **(c)** study types in ToxRefDB (DEV: Developmental, MGR: multigeneration, DNT: developmental neurotoxicity or NEU: neurotoxicity), zebrafish (morphology, mortality, EPR or LPR) and planarian assay endpoints (morphology, mortality, early and late behavior). Each color represents one of these parameters, and the line indicates the relationship between two parameters. The width of each parameter is a count of the number of relationships. Thus, a relatively longer width represents that chemical/endpoint has more identified links to the other chemical/endpoints.

Discussion

Trends by chemical class

Differences were observed in the sensitivity of the two systems to the various chemical classes in the NTP 87-compound library. Since almost all chemicals were bioactive in the zebrafish screen, concordance was based on whether a zebrafish hit was also a planarian hit. Concordance (from most to least): pesticides (15/16, planarian/zebrafish; 94%), flame retardants (10/15, 67%), drugs (10/18, 56%), industrial chemicals (7/15, 47%), and PAHs (6/17, 35%). The class of PAHs had the lowest concordance between the two models, which may be due to the absence of known PAH targets and pathways in planarians. Some PAHs activate the aryl hydrocarbon receptor (AHR) to produce toxicity and cancer (Choi et al., 2010; Garcia et al., 2018; Geier et al., 2018; Knecht et al., 2017b; Qiao et al., 2017). Benzo[a]pyrene, a hit in zebrafish, but not planarians, produces developmental and neurobehavioral deficits dependent on the presence of the AHR2 (Incardona et al., 2011; Knecht et al., 2017a). Thus, lack of conservation of the AHR pathway in planarians may explain the observed insensitivity to PAH exposure.

Molecular weight, log K_{ow}, and BCF values are physicochemical properties proposed to be the most predictive for water exposure. In this study, we found that not to be true as no clear trends emerged for actives and inactives in the planarian system (Figure 4.4). As 86 of the 87 chemicals were hits in the zebrafish model, it was not feasible to assess this trend. However, the one negative, hydroxyurea, did not have any extreme values, supporting the conclusion from the planarian system that the 3 parameters are weakly predictive of bioactivity.

Differences in chemical sensitivity could be due to a variety of factors: route of exposure/ chemical uptake, metabolic activity, etc. It is worth noting that while exposure in both systems is

mainly achieved through epidermal diffusion, other routes (e.g. planarian pharynx) can also be involved, the extent to which may depend on the life-stage of the animal or the chemical itself. Additionally, planarians are covered in a protective mucus barrier, important for defense against infection and injury (Cochet-Escartin et al., 2015; Pedersen, 2008), which may impede uptake of some chemicals.

The NTP 87-compound library curators classified 5 chemicals as inactive in the toxicological screens performed to date under the range of test conditions used: Acetaminophen, acetylsalicylic acid (aspirin), D-glucitol, L-ascorbic acid and saccharin sodium salt hydrate. Two of these (acetaminophen and acetylsalicylic acid) were identified as hits in animal guideline studies (MGR and DEV, respectively). Additionally, they were also found to be bioactive in the zebrafish LPR and in regenerating planarian late behavior (acetaminophen) and morphology and early/late behaviors (acetylsalicylic acid). However, we note that the regenerating planarian behavioral effects of acetaminophen were very mild, being just outside the noise level (biological cutoffs) of the controls (Zhang et al., 2018). Other studies have also observed this bioactivity (Marques et al., 2004; Prášková et al., 2012; Weigt et al., 2010). Both the zebrafish and planarian detected bioactivity for these 2 misclassified DNTs and did so in under 12 days. The remaining 3 NTP-inactives had either limited data (D-glucitol), were a developmental toxicant (L-ascorbic acid), or a known carcinogen (saccharin sodium salt hydrate) (https://sandbox.ntp.niehs.nih.gov/neurotox/). Although these 3 chemicals were not DNT compounds, they were bioactive in the zebrafish assays, likely due to the sensitivity of the developing zebrafish as a biosensor and the fact that highly diverse chemical insults during vertebrate development often manifest as common endpoint readouts. Of note, none of the negative controls were active in adult planarians (Supplementary Figure 3). However, it is

difficult to classify chemicals as being negative when dosimetry is unknown, in any model system. Therefore, the differences in classification could be due to different databases and criteria.

The battery of models approach to screening and its predictive power

Whatever the end goal of a chemical screen might be, the principles of the 3Rs (Replacement, Reduction, and Refinement (Dix et al., 2007)) and good scientific practice collectively necessitate that the simplest yet most informative model that minimizes the number of false negatives and false positives is the preferred choice. However, in reality, no one model is likely to be sufficient to capture all necessary biological space in a time and cost-efficient manner. Thus, battery testing relying on comparative analysis across a range of complementary models (including both *in vitro* and *in vivo* systems) may provide the best option for efficient testing, particularly during early hazard identification and prioritization.

In this study, we showed that the zebrafish and planarian models provide a complementary assessment of biological space making them well-suited for battery-approach screening. The optical transparency of developing zebrafish allows for a wide range of morphological assessments to monitor proper developmental milestones and organ formation, exemplified by the high concordance of bioactivity in zebrafish morphological endpoints and ToxRefDB DEV studies. Additionally, their rapid development allows for integration of the central nervous system and assessment for developmental toxicants. On the other hand, the breadth of quantifiable planarian behaviors, some of which are known to be controlled through distinct neuronal subpopulations (Currie and Pearson, 2013; Inoue et al., 2014; Nishimura et al., 2010), provides insight in the mechanisms of (developmental) neurotoxicity. Moreover,

planarians are uniquely suited to allow for direct comparisons between adult and developing animals to be able to distinguish developmental effects from general (neuro-)toxicity. In fact, we found that 13 of the 50 chemicals active in regenerating planarians were developmentally selective, i.e. toxicity was not found in adult planarians or was found at a higher concentration (Zhang et al., 2018).

Moreover, the zebrafish and planarian models were concordant in their bioactivity readouts across the diverse chemical space captured in this NTP 87-compound library (Figure 4.3). When comparing actives in the different endpoint classes, both systems contained hits that were not captured by the other. Most chemicals of this library were bioactive in the developing zebrafish, which may be due to the large number of morphological endpoints evaluated. It may appear that the zebrafish model is oversensitive, as it identified 86/87 (98.8%) chemicals as bioactive and some of these hits may be false positives. However, the NTP 87-compound library was specifically designed to consist primarily of developmental, developmental neurotoxic, or neurotoxic chemicals, and the zebrafish early life stage system is sensitive to all of these defined endpoints, plus others, such as carcinogenicity. Thus, the high hit rate may be a direct consequence of this particular library. The unique endpoint hits in planarians (particularly late behavior, consisting of 4 different endpoints testing both stimulated (in response to light, thermal gradients and noxious heat stimuli) and unstimulated behaviors) may provide additional insight into the phenotypic profiling and mechanisms of neurotoxicity for some chemicals.

Even combined, the zebrafish and planarians models are likely not sufficient to capture all realms of possible human health hazards. For example, both models are aquatic organisms relying on chemical exposure in their aquatic environment. This may lead to inconsistencies in toxic outcomes when compared to the breadth of possible routes of exposure in other systems.

Moreover, the relationship between the nominal chemical concentrations and the internal concentrations found in the animals is often lacking and could be affected by various factors (solubility issues, absorption by the plastic, absorption into the animal, instability in water over the course of the screen, metabolism, etc.). The understanding of these factors and the pharmacokinetic/pharmacodynamics in these systems will be essential for further validation. This will be particularly important to compare and connect activity in these models with the relevant concentrations/exposure seen in mammals and humans.

The appropriateness and effectiveness of gold standard in vivo mammalian, developmental neurotoxicity studies, which can take 130+ days from exposure to evaluation of the neurobehavioral development of the offspring through adulthood (Dubovický et al., 2008; Virginia Moser et al., 2016) is fiercely debated. The time and expense costs of such guideline studies make them inadequate to evaluate the growing list of chemicals of concern (Tsuji and Crofton, 2012). Moreover, there is uncertainty how to accurately extrapolate data from rats to humans. By adapting high throughput alternative models, we can streamline the toxicology pipeline to efficiently prioritize which chemicals should be tested in guideline studies. These alternative models will likely not completely replace guideline studies, which may still be required for decision making, but can provide rapid guidance of which studies are worth pursuing and which toxicants are of the greatest concern. Libraries such as the NTP 87-compound library tested here, which are enriched in chemicals with known toxicity, are useful tools for model validation to determine whether effects in alternative models are predictive of mammalian, and ultimately, human toxicity. Twenty-seven of the 28 compounds (96%) in this library which had quality guideline studies associated with them in ToxRefDB were bioactive in either the planarian and/or zebrafish screens, with 20 (71%) bioactive in both, validating the

predictivity and relevancy of these models for mammalian toxicity. Using both systems, we are able to provide necessary information to prioritize the chemicals of highest concern, such as in (Behl et al., 2015), and help fill the data gaps of under-represented toxicant classes with potential hazards in a relatively quick manner. We thus envision using the zebrafish and planarian models as primary screening tools for vast swathes of chemical space, building big structure-bioactivity datasets from which to prioritize chemicals for further evaluation in the current testing pipeline and potentially predict chemical hazard in the future.

Thus, while a lot of work remains to be done to understand how these and other alternative systems compare to the standard toxicology models, what this and the other studies of the same chemical library in this special issue demonstrate, is the added value alternative models are bringing to modern toxicology. A battery approach that harvests the strengths of each of these systems in combination will ultimately transform the toxicology pipeline.

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Chapter 4, in full, is a reformatted reprint of the material as it appears Toxicological Sciences 2018 (Hagstrom, Danielle; Truong, Lisa; Zhang, Siqi; Tanguay, Robert L; and Collins, Eva-Maria S. "Comparative analysis of zebrafish and planarian model systems for developmental neurotoxicity screens using an 87-compound library", Toxicological Sciences 2018). The version of record is available online at: https://academic.oup.com/toxsci/advance-article-abstract/doi/10.1093/toxsci/kfy180/5053695. Use of this manuscript in the dissertation herein is covered by the rights permitted to the authors by Oxford Journals. The dissertation author was the co-author of this paper. The original planarian screening data was obtained by Siqi Zhang, Danielle Hagstrom and Eva-Maria S. Collins as described in Chapter 3. Lisa Truong and Robert Tanguay designed, executed, and analyzed the experiments associated with the zebrafish screening data. Danielle Hagstrom performed the direct screening result comparisons while Lisa Truong performed the comparisons with available mammalian data and physico-chemical properties. We thank Christina Rabeler for help with data compilation, and the staff at Oregon State University Sinnhuber Aquatic Research Laboratory for their assistance in the zebrafish screening. Danielle Hagstrom and Lisa Truong were the primary investigators and authors of this material.

References

Bailey, J., Oliveri, A. and Levin, E. D. (2013). Zebrafish model systems for developmental neurobehavioral toxicology. Birth Defects Res. C. Embryo Today 99, 14–23.

Behl, M., Hsieh, J. H., Shafer, T. J., Mundy, W. R., Rice, J. R., Boyd, W. A., Freedman, J. H., Hunter, E. S., Jarema, K. A., Padilla, S. and Tice, R.R. (2015). Use of alternative assays to identify and prioritize organophosphorus flame retardants for potential developmental and neurotoxicity. Neurotoxicol. Teratol.

Behl, M., Ryan, K., Hsieh, J.-H., Parham, F., Shapiro, A., Collins, B. J., Birnbaum, L. S., Bucher, J. R., Walker, N. J., Foster, P. M., et al. (2018). Screening for developmental neurotoxicity (DNT) at the National Toxicology Program: The future is now! Toxicol. Sci.

Boyd, W., Smith, M. and Freedman, J. (2012). Caenorhabditis elegans as a model in developmental toxicology. In Developmental Toxicology (ed. Harris, C.) and Hansen, J. M.), pp. 15–24. Totowa, NJ: Humana Press.

Boyd, W. A., Smith, M. V., Co, C. A., Pirone, J. R., Rice, J. R., Shockley, K. R. and Freedman, J. H. (2015). Developmental effects of the ToxCastTM phase I and phase II chemicals in Caenorhabditis elegans and corresponding responses in zebrafish, rats, and rabbits. Environ. Health Perspect. 124, 586–93.

Cebrià, F. (2007). Regenerating the central nervous system: how easy for planarians! Dev. Genes Evol. 217, 733–48.

Choi, H., Harrison, R., Komulainen, H. and Delgado Saborit, J. M. (2010). Polycyclic aromatic hydrocarbons. In WHO guidelines for indoor air quality: selected pollutants, pp. 289–345. Bonn, Germany: World Health Organization.

Cochet-Escartin, O., Mickolajczk, K. J. and Collins, E.-M. S. (2015). Scrunching: a novel escape gait in planarians. Phys. Biol. 12, 055001.

Currie, K. W. and Pearson, B. J. (2013). Transcription factors lhx1/5-1 and pitx are required for the maintenance and regeneration of serotonergic neurons in planarians. Development 140, 3577–88.

Dix, D. J., Houck, K. A., Martin, M. T., Richard, A. M., Setzer, R. W. and Kavlock, R. J. (2007). The Toxcast program for prioritizing toxicity testing of environmental chemicals. Toxicol. Sci. 95, 5–12.

Dubovický, M., Kovačovský, P., Ujházy, E., Navarová, J., Brucknerová, I. and Mach, M. (2008). Evaluation of developmental neurotoxicity: some important issues focused on neurobehavioral development. Interdiscip. Toxicol. 1, 206–10.

Garcia, G. R., Bugel, S. M., Truong, L., Spagnoli, S. and Tanguay, R. L. (2018). AHR2 required for normal behavioral responses and proper development of the skeletal and reproductive

systems in zebrafish. PLoS One 13, e0193484.

Geier, M. C., Chlebowski, A. C., Truong, L., Massey Simonich, S. L., Anderson, K. A. and Tanguay, R. L. (2018). Comparative developmental toxicity of a comprehensive suite of polycyclic aromatic hydrocarbons. Arch. Toxicol. 92, 571–586.

Hagstrom, D., Cochet-Escartin, O., Zhang, S., Khuu, C. and Collins, E.-M. S. (2015). Freshwater planarians as an alternative animal model for neurotoxicology. Toxicol. Sci. 147, 270–285.

Hagstrom, D., Cochet-Escartin, O. and Collins, E.-M. S. (2016). Planarian brain regeneration as a model system for developmental neurotoxicology. Regeneration 3, 65–77.

He, J.-H., Gao, J.-M., Huang, C.-J. and Li, C.-Q. (2014). Zebrafish models for assessing developmental and reproductive toxicity. Neurotoxicol. Teratol. 42, 35–42.

Hill, A. J., Teraoka, H., Heideman, W. and Peterson, R. E. (2005). Zebrafish as a model vertebrate for investigating chemical toxicity. Toxicol. Sci. 86, 6–19.

Howe, K., Clark, M. D., Torroja, C. F., Torrance, J., Berthelot, C., Muffato, M., Collins, J. E., Humphray, S., McLaren, K., Matthews, L. and McLaren, S. (2013). The zebrafish reference genome sequence and its relationship to the human genome. Nature 496, 498–503.

Incardona, J. P., Linbo, T. L. and Scholz, N. L. (2011). Cardiac toxicity of 5-ring polycyclic aromatic hydrocarbons is differentially dependent on the aryl hydrocarbon receptor 2 isoform during zebrafish development. Toxicol. Appl. Pharmacol. 257, 242–249.

Inoue, T., Yamashita, T. and Agata, K. (2014). Thermosensory signaling by TRPM is processed by brain serotonergic neurons to produce planarian thermotaxis. J. Neurosci. 34, 15701–14.

Knecht, A. L., Truong, L., Simonich, M. T. and Tanguay, R. L. (2017a). Developmental benzo[a]pyrene (B[a]P) exposure impacts larval behavior and impairs adult learning in zebrafish. Neurotoxicol. Teratol. 59, 27–34.

Knecht, A. L., Truong, L., Marvel, S. W., Reif, D. M., Garcia, A., Lu, C., Simonich, M. T., Teeguarden, J. G. and Tanguay, R. L. (2017b). Transgenerational inheritance of neurobehavioral and physiological deficits from developmental exposure to benzo[a]pyrene in zebrafish. Toxicol. Appl. Pharmacol. 329, 148–157.

Linney, E., Upchurch, L. and Donerly, S. (2004). Zebrafish as a neurotoxicological model. In Neurotoxicology and Teratology, pp. 709–718.

Maes, J., Verlooy, L., Buenafe, O. E., de Witte, P. A. M., Esguerra, C. V and Crawford, A. D. (2012). Evaluation of 14 organic solvents and carriers for screening applications in zebrafish embryos and larvae. PLoS One 7, e43850.

Malinowski, P. T., Cochet-Escartin, O., Kaj, K. J., Ronan, E., Groisman, A., Diamond, P. H. and Collins, E.-M. S. (2017). Mechanics dictate where and how freshwater planarians fission. Proc. Natl. Acad. Sci. U. S. A. 114, 10888–10893.

Marques, C. R., Abrantes, N. and Gonçalves, F. (2004). Life-history traits of standard and autochthonous cladocerans: I. Acute and chronic effects of acetylsalicylic acid. Environ. Toxicol. 19, 518–526.

Nishimura, K., Kitamura, Y., Taniguchi, T. and Agata, K. (2010). Analysis of motor function modulated by cholinergic neurons in planarian Dugesia japonica. Neuroscience 168, 18–30.

Noyes, P. D., Haggard, D. E., Gonnerman, G. D. and Tanguay, R. L. (2015). Advanced morphological-behavioral test platform reveals neurodevelopmental defects in embryonic zebrafish exposed to comprehensive suite of halogenated and organophosphate flame retardants. Toxicol. Sci. 145, 177–195.

Paskin, T. R., Jellies, J., Bacher, J. and Beane, W. S. (2014). Planarian phototactic assay reveals differential behavioral responses based on wavelength. PLoS One 9, e114708.

Pedersen, K. J. (2008). Sliime-secreting cells of planarians. Ann. N. Y. Acad. Sci. 106, 424–443.

Prášková, E., Živná, D., Štěpánová, S., Ševčíková, M., Blahová, J., Maršálek, P., Široká, Z., Voslářová, E. and Svobodová, Z. (2012). Acute toxicity of acetylsalicylic acid to juvenile and embryonic stages of Danio rerio. Neuroendocrinol. Lett. 33, 72–76.

Qiao, Y., Li, Q., Du, H.-Y., Wang, Q.-W., Huang, Y. and Liu, W. (2017). Airborne polycyclic aromatic hydrocarbons trigger human skin cells aging through aryl hydrocarbon receptor. Biochem. Biophys. Res. Commun. 488, 445–452.

R Core Team (2016). R: A language environment for statistical computing. Vienna, Austria.

Reif, D. M., Truong, L., Mandrell, D., Marvel, S., Zhang, G. and Tanguay, R. L. (2016). High-throughput characterization of chemical-associated embryonic behavioral changes predicts teratogenic outcomes. Arch. Toxicol. 90, 1459–1470.

Rink, J. C. (2013). Stem cell systems and regeneration in planaria. Dev. Genes Evol. 223, 67–84.

Ryan, K. R., Sirenko, O., Parham, F., Hsieh, J. H., Cromwell, E. F., Tice, R. R. and Behl, M. (2016). Neurite outgrowth in human induced pluripotent stem cell-derived neurons as a high-throughput screen for developmental neurotoxicity or neurotoxicity. Neurotoxicology 53, 271–281.

Tejeda-Benitez, L. and Olivero-Verbel, J. (2016). Caenorhabditis elegans, a biological model for research in toxicology. In Reviews of Environmental Contamination and Toxicology (ed. de Voogt, W.), pp. 1–35. Springer, Cham.

Truong, L., Reif, D. M., St Mary, L., Geier, M. C., Truong, H. D. and Tanguay, R. L. (2014).

Multidimensional in vivo hazard assessment using zebrafish. Toxicol. Sci. 137, 212–33.

Truong, L., Bugel, S. M., Chlebowski, A., Usenko, C. Y., Simonich, M. T., Simonich, S. L. M. and Tanguay, R. L. (2016). Optimizing multi-dimensional high throughput screening using zebrafish. Reprod. Toxicol. 65, 139–147.

Tsuji, R. and Crofton, K. M. (2012). Developmental neurotoxicity guideline study: Issues with methodology, evaluation and regulation. Congenit. Anom. (Kyoto). 52, 122–128.

Virginia Moser, Raffaele, K., Crofton, K., Gilbert, M., Bowers, W. and Bailey, F. (2016). NAFTA Technical working group on pesticides (twg): Developmental neurotoxicity study (DNT) guidance document.

Weigt, S., Huebler, N., Braunbeck, T., von Landenberg, F. and Broschard, T. H. (2010). Zebrafish teratogenicity test with metabolic activation (mDarT): Effects of phase I activation of acetaminophen on zebrafish Danio rerio embryos. Toxicology 275, 36–49.

Zhang, G., Roell, K. R., Truong, L., Tanguay, R. L. and Reif, D. M. (2017). A data-driven weighting scheme for multivariate phenotypic endpoints recapitulates zebrafish developmental cascades. Toxicol. Appl. Pharmacol. 314, 109–117.

Zhang, S., Hagstrom, D., Hayes, P., Graham, A. and Collins, E.-M. S. (2018). Multi-behavioral endpoint testing of an 87-chemical compound library in freshwater planarians. Toxicol. Sci.

Chapter 5. Analysis of the concordance and robustness of the freshwater planarian neurotoxicology model using 15 flame retardants

Abstract

Alternative methods, including *in vitro* and non-mammalian animal models, amenable to rapid and cost-effective screening, are emerging to fill the urgent need to strategically accelerate the hazard assessment of existing and new environmental chemicals. Among these alternative animal models are asexual freshwater planarians, which are particularly well-suited for assessing developmental neurotoxicity (DNT), because they allow for a direct comparison of adult and regenerating/developing animals with the same assays. Additionally, planarians possess a large repertoire of quantifiable behaviors, enabling phenotypic readouts of neuronal function. In this study, we used a fully automated planarian screening platform to screen and assess 15 flame retardants (FRs), consisting of both brominated (BFR) and organophosphorous (OPFR) FRs, for potential (developmental) neurotoxicity. We find 11 of the 15 FRs (4/7 BFRs and 7/8 OPFRs) were active in both adult and regenerating planarians. By comparing our data with previously published data in zebrafish, nematode and *in vitro* cell-based models, we show that the planarian model has high concordance and comparable sensitivity to other alternative models. Furthermore, as this FR chemical set was a subset of a 87-compound library provided by the National Toxicology Program (NTP), which we previously screened (Zhang et al., 2018), we took this opportunity to evaluate the robustness and reproducibility of our planarian platform by comparing the results of different numbers of replicates (from 3-6) from these two independent screens. We found that 3 replicates (n=24 planarians per chemical per concentration) yielded the exact same chemical hit list as 6 replicates (n=48 planarians per chemical per concentration) and only observed minor differences in 4% of all endpoint readouts. This result demonstrates that the performance of 3 replicates are a robust and efficient screening strategy in our system.

Introduction

Traditional toxicity assessment using mammalian models cannot keep pace with the speed of chemical development as thousands of compounds already prevalent in the environment are not adequately evaluated for all potential toxicity, particularly for effects on development (Bennett et al., 2016). Hence, alternative *in vitro* and non-mammalian models, amenable to rapid, cost-effective screening, have been established to accelerate hazard assessment and complement mammalian models. While each system has its own advantages and drawbacks, a battery approach using medium- and high-throughput screening (MTS and HTS) in multiple complementary models provides greater weight of evidence for prioritization of further evaluation in mammalian models (Behl et al., 2015; Behl et al., 2018) .

Our laboratory has pioneered the use of the asexual freshwater planarian, *Dugesia japonica,* as a MTS alternative animal model to study developmental neurotoxicity (DNT) and have shown this system to complement established alternative animal models, such as zebrafish and nematodes (Hagstrom et al.; Hagstrom et al., 2015; Zhang et al., 2018). Besides low cost, small size, and rapid development, the planarian system has the unique advantage to study adult and regenerating/developing animals in parallel with the same assays (Hagstrom et al., 2015; Hagstrom et al., 2016; Zhang et al., 2018). Moreover, while offering limited morphological endpoints compared to zebrafish, the planarian system possesses a larger repertoire of different quantifiable behaviors (e.g. phototaxis, thermotaxis, pain response), allowing for assessment of specific neuronal functions to provide insight into the mechanisms of neurotoxicity (Hagstrom et al.). Because of its relative novelty as a toxicology model, findings in planarians must be compared to more established systems and validated for concordance, relevancy, and robustness to determine how planarians could fit into the modern toxicological pipeline. We recently began

this validation process using an 87-compound library of known and suspected developmental neurotoxicants provided by the National Toxicology Program (NTP) (Behl et al., 2018; Hagstrom et al.; Zhang et al., 2018). Here, we focus on a subset of these compounds, namely FRs and perform a second, independent screen of the FRs. Comparing the results with the NTP-library screen, we assess the robustness of our planarian system. We find that our current strategy of testing 3 replicates per compound per concentration, consisting of n=24 full or regenerating planarians, respectively, is sufficient to robustly identify chemical hits.

FRs provide a well-suited, discrete set of environmentally important chemicals by which to further evaluate the concordance and relevancy of the planarian system, because they have been studied in other alternative and (to a lesser extent) mammalian models (Behl et al., 2015; Costa and Giordano, 2007; Jarema et al., 2015; Noyes et al., 2015). FRs are widely added to commercial products for fire safety(Costa and Giordano, 2007; Salimi et al., 2017). Prior to 2005, polybrominated diphenyl ether (PBDE) mixtures were the primary FRs used in the United States (Hale et al., 2003), but many were voluntarily phased-out due to growing evidence of their association with impaired neurodevelopment and decreased fertility, exacerbated by their persistence in the environment and ability to bioaccumulate (Darnerud, 2003; Herbstman et al., 2010; Stapleton et al., 2009a; Stapleton et al., 2011; Talsness, 2008). OPFRs have emerged as replacements for PBDEs (Stapleton et al., 2012; Stapleton et al., 2014). However, little is known about their potential toxicity, although OPFRs were suggested to persist in the environment and bioaccumulate, similar to PBDEs (Meeker and Stapleton, 2010; Stapleton et al., 2009b; Van Der Veen and De Boer, 2012). Potential DNT of OPFRs is of particular concern, as OPFRs share structural similarities to organophosphorous pesticides, which have been shown to adversely affect neurodevelopment and cause neurobehavioral impairments (González-Alzaga et al., 2014;

Muñoz-Quezada et al., 2013; Ricceri et al., 2006; Slotkin et al., 2006). A direct comparison of a battery screen of 14 FRs found that most OPFRs had comparable activity to brominated FR (BFRs), including the phased-out BDE-47 (Behl et al., 2015).

Thus, herein, we assess the potential (developmental) neurotoxicity of 15 FRs (Table 5.1), including 3 phased-out PBDEs (BDE-47, BDE-99 and BDE-153), 3 currently in-use BFRs (TBB, TBBPA, and TBPH), 8 in-use OPFRs (BPDP, EHDP, IDDP, IPP, TCEP, TCPP, TMPP, and TPHP), and one BFR/OPFR mixture (Firemaster 550) using our automated planarian MTS platform, utilizing morphological and behavioral endpoints. We find that 11 of the 15 FRs were active in both adult and regenerating planarians. We compared our results with published data in zebrafish, nematode and human, mouse and rat cell culture models (Behl et al., 2015; Behl et al., 2016; Jarema et al., 2015; Noyes et al., 2015) and find high concordance of activity and similar levels of sensitivity between planarians and these systems.

Together, our results illustrate the planarian's value in the modern toxicology pipeline, especially in a battery approach, utilizing the strengths of various models.

Material and methods

Chemical library

18 chemicals consisting of 6 brominated flame retardants (BFRs, including phased-out BDE-47 and BDE-99), 8 OPFRs, 1 BFR/OPFR mixture, 2 negative controls and 1 duplicate (TPHP) were screened in this study. Table 5.1 lists the 17 unique chemicals with their name, chemical ID, type, structure, supplier, and purity. The chemicals were provided at ~ 20mM stocks in dimethyl sulfoxide (DMSO, Gaylord Chemical Company, Slidell, LA), with the exception of 2,2',4,4',5,5'-Hexabromodiphenyl ether (BDE-153), which was provided at 10mM. The chemicals were tested at 0.01, 0.1, 1, 10 and 100μM (0.005, 0.05, 0.5, 5 and 50μM for BDE-153), with a final DMSO concentration of 0.5% for all concentrations. Solvent control populations were also exposed to 0.5% DMSO. The tested FRs were a subset of the NTP 87-compound library, which we had screened in its entirety (FR screen 1) and previously reported the results (Zhang et al.). We were later provided with a duplicate set of the library (now including 4 duplicate chemicals for a total of 91 chemicals), in which we only screened the subset of chemicals mentioned in this study (FR screen 2). Chemicals in the new library were provided as individual vials and stored at 4ºC. Otherwise, chemicals were prepared and stored as described previously (Zhang et al., 2018). Additional information on the NTP library can be found in (Behl et al., 2018).

Table 5.1. Summary of the screened chemical library with CAS number, chemical name, ID, type (BFR: brominated flame retardant, OPFR: organophosphate flame retardant), chemical supplier, structure, and purity.

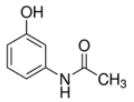
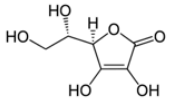
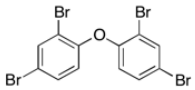
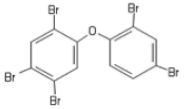
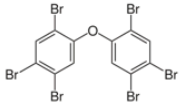
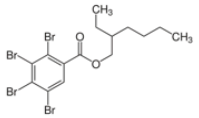
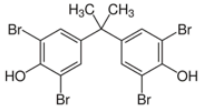
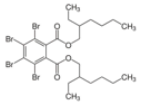
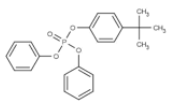
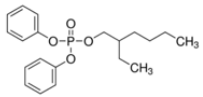
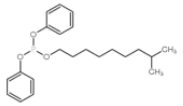
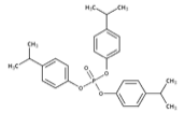
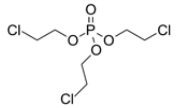
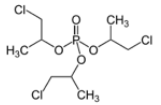
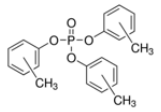
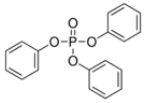
CASRN	Chemical name	ID	Type	Suppliers	Structure	Purity
103-90-2	Acetaminophen (4-hydroxyacetanilide)		Negative control	Sigma-Aldrich		100%
50-81-7	L-Ascorbic acid		Negative control	Sigma-Aldrich		100%
5436-43-1	2,2',4,4'-Tetrabromodiphenyl ether	BDE-47	BFR	Cerilliant Corp.		100%
60348-60-9	2,2',4,4',5-Pentabromodiphenyl ether	BDE-99	BFR	Cerilliant Corp.		100%
68631-49-2	2,2',4,4',5,5'-Hexabromodiphenyl ether	BDE-153	BFR	Cerilliant Corp. via Battelle Memorial Institute		100%
183658-27-7	2-ethylhexyl-2,3,4,5-tetrabromobenzoate	TBB	BFR	Toronto Research Chemicals		NA
79-94-7	3,3',5,5'-Tetrabromobisphenol A	TBBPA	BFR	Sigma-Aldrich		99.77%
26040-51-7	Bis(2-ethylhexyl) 3,4,5,6-tetrabromophthalate	TBPH	BFR	Toronto Research Chemicals		NA
860302-33-6	Firemaster 550		mixture of BFR and OPFR	Chemtura Corporation		NA

Table 5.1. Summary of the screened chemical library with CAS number, chemical name, ID, type (BFR: brominated flame retardant, OPFR: organophosphorous flame retardant), chemical supplier, structure, and purity (continued).

CASRN	Chemical name	ID	Type	Suppliers	Structure	Purity
56803-37-3	tert-Butylphenyl diphenyl phosphate	BPDP	OPFR	Ubichem PLC via MRIGlobal		98.22%
1241-94-7	2- ethylhexyl diphenyl phosphate	EHDP	OPFR	TCI America		98.67%
29761-21-5	Isodecyl diphenyl phosphate	IDDP	OPFR	Bayville Chemical Supply Company Inc.		92.19%
68937-41-7	Phenol, isopropylated, phosphate (3:1)	IPP	OPFR	Amfinecom Inc.		NA
115-96-8	Tris(2-chloroethyl) phosphate	TCEP	OPFR	Sigma-Aldrich		99.07%
13674-84-5	tris(2-Chloroisopropyl)phosphate	TCPP	OPFR	Albemarle Corporation via MRIGlobal		99.47%
1330-78-5	Tricresyl phosphate	TMPP	OPFR	Acros Organics		100%
115-86-6	Triphenyl phosphate	TPHP	OPFR	Sigma-Aldrich		100%

† Firemaster 550 is a chemical mixture, comprised of 2-ethylhexyl-2,3,4,5-tertrabromobenzoate (TBB), bis(2-ethylhexyl) tetrabromophthalate (TBPH), triphenyl phosphate (TPP), and isopropylated triphenyl phosphate (IPTP) (Tung et al., 2017)

FR screen in the planarian system

Asexual *D. japonica*, originally obtained from Shanghai University (Shanghai, China) and cultivated in our lab for > 5 years, were used for all experiments. Briefly, we screened the library with a fully automated planarian MTS platform using full (intact) and regenerating (tail pieces) planarians in parallel with the same assays, assessing both morphological and behavioral endpoints at day 7 and day 12 (Figure 5.1). Two independent screens, the original NTP 87-compound library screen ("FR screen 1") and the second, FR-only screen ("FR screen 2"), were performed. We applied the same experimental procedures (Figure 5.1) and data analysis methods as described in detail in (Zhang et al., 2018), except for the following differences in FR screen 2, building upon our experiences from FR screen 1: Firstly, since we found that food quality affects planarian fitness and sensitivity to chemicals (Zhang et al., 2018), we fed planarians used in FR screen 2 commercial freeze-dried organic chicken liver (Amazon, Seattle, WA) to better control food quality and thus minimize animal fitness variability. Secondly, "biological relevance cutoffs", used to minimize false positives by accounting for variability of the solvent controls in each endpoint (Zhang et al., 2018), were updated when comparing both screens by using the combined DMSO control populations from the previous NTP 87-compound screen (n=87 control populations) and the later FR-only screen (n=18 control populations) for a total control group of n=105 populations, each consisting of n=24 planarians. Thirdly, the background noise speed threshold used in the phototaxis assay was recalculated based on the mean speed in the unstimulated behavioral assay of the expanded DMSO controls.

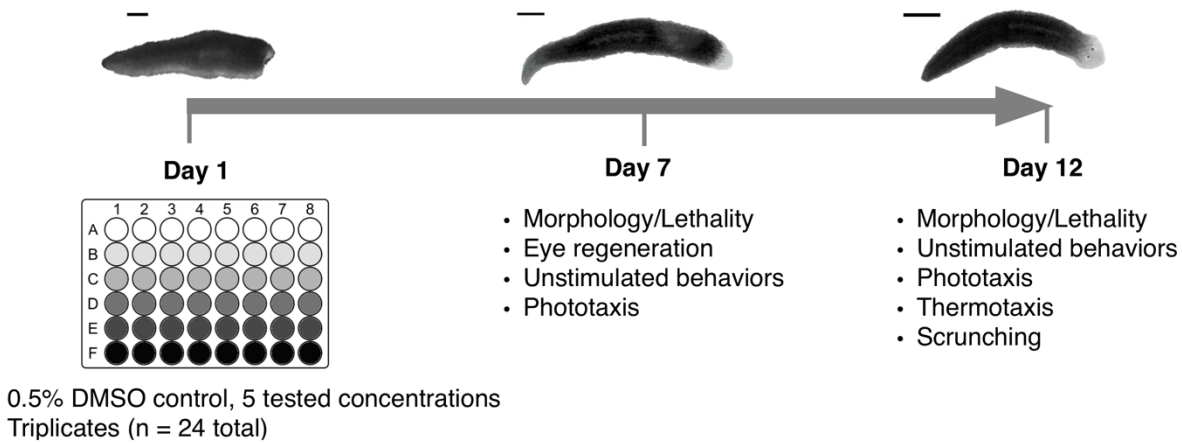


Figure 5.1. Schematic of overall screen flow in the planarian system. Exposure lasted 12 days. Planarians were amputated and set up into 48-well plates with chemicals at day 1, and assayed with different morphological and behavioral endpoints at day 7 and day 12. Scale bar: 1mm.

Comparison to published literature

The planarian data in this study was compared to the published data in nematode *C. elegans*, zebrafish and *in vitro* (mouse embryonic stem cells, human neural stem cells, and rat neurons) systems (Behl et al., 2015; Behl et al., 2016; Jarema et al., 2015; Noyes et al., 2015). Comparisons were made with the lowest effect level (LEL) determined in planarians from the compiled data of 6 replicate runs from FR screens 1 and 2. Of note, the activity of the compounds was assessed in the different models in different manners, either LEL or point of departure (POD) calculations. In the two zebrafish published papers (Jarema et al., 2015; Noyes et al., 2015), LELs they reported included concentration-independent and hyperactivity effects. But for the purpose of model comparison, we do not considerate concentration-independent and hyper-activity effects for LELs. Effects that were seen at lower concentrations but not higher concentrations were considered concentration-independent.

Robustness analysis of planarian screening platform

Six replicates from the two independent flame retardant screens, FR screen 1 and 2, were used to evaluate the robustness of the planarian screening platform. Because the level of toxicity of many of these FRs is unknown in planarians (as well as overall), we do not have an objective metric to compare our results to. It is reasonable, however, to assume that using all 6 replicates provides the most accurate results given the large sample size (n=48). We therefore compared the results of compiling different numbers of replicates (3, 4 or 5) with the results obtained by compiling 6 replicates. As described previously (Zhang et al., 2018), we used a 3-step (statistical test, biological relevance cutoff and inconsistency check) statistical workflow to determine whether a chemical showed bioactivity in any endpoint. In the following, we refer to bioactivity per endpoint as “readout” to distinguish it from a bioactive “hit”, which refers to having an effect

at any endpoint. Since the biological relevance cutoffs are meant as a means to understand normal wild-type noise levels, biological relevance cutoffs were determined based on the total control group of n=105 control sets (see section of FR screen in the planarian system), and were applied equally to these different sets of compiled data (i.e. the same cutoffs, determined from this total control group, were used for 3, 4, 5, or 6 replicates). For the inconsistency check, instances were excluded where more than half of a set of replicates (i.e. >1 out of 3 replicates, >2 out of 4 replicates, >2 out of 5 replicates, >3 out of 6 replicates) were inside of the biological relevancy cutoffs. Although the majority of the experimental procedures were consistent between the two screens some differences existed, which could affect the reproducibility of the screens. For example, varying food quality (between different home-made batches and between home-made and commercial sources) affects animal health and thus affect the animals' sensitivity to chemical exposure (Zhang et al., 2018). Different chemical batches and purities can also cause variability due to differences in stock concentrations or handling of the chemicals. Therefore, we chose replicates with the most similar conditions (i.e. in the same screen) to minimize experimental variability and to focus on the robustness of the system itself. To compile 3 replicates, we chose the 3 replicates from FR screen 2. To compile 4 (or 5) replicates, we chose 1 (or 2) replicate(s) from FR screen 1 in addition to the 3 replicates from FR screen 2. Firstly, overall chemical bioactivity (i.e. whether the chemical was bioactive in any endpoint or inactive in all endpoints) in different sets of replicates (3, 4, 5 or 6) was compared. Secondly, concordance of the bioactivity of the readouts (i.e. whether the chemical was deemed bioactive or inactive in a specific endpoint) between the data using 3, 4 or 5 replicates and the data using 6 replicates were compared. Lastly, the concordance of the sensitivity of the concordant bioactive

readouts (i.e. the LEL of the bioactive readout) was compared between the data using 3, 4 or 5 replicates and the data using 6 replicates.

Results and discussion

Summary of planarian FR screen

A library of 15 FRs and 2 negative controls (acetaminophen and L-ascorbic acid) (Table 5.1), was screened in adult and regenerating planarians to assess for effects on mortality, eye regeneration and neuronal activity. These chemicals were screened twice, once as part of the NTP 87-compound library screen (FR screen 1) (Zhang et al., 2018) and once as a new screen of the FR-specific subset of a newly obtained version of the library (FR screen 2), with 3 replicates performed in each screen (total n=24 per screen). Data of the 6 replicates was used for further evaluation of the system's variability and robustness.

Considering all compiled data, of the 15 tested FRs, 11 were bioactive in both full and regenerating planarians at nominal concentrations of 10-100 μM in at least 1 endpoint (Figure 5.2). The bioactive FRs consisted of 3 of the 6 BFRs (BDE-99, BDE-47, TBBPA,), 7 of the 8 OPFRs (IDDP, TCPP, EHDP, IPP, BPDP, IPP, BPDP, TMPP) and the FM550 mixture. TBBPA was the most potent BFR with a LEL of 10μM in multiple endpoints in both regenerating and full planarians. The OPFRs generally showed higher potency than the BFRs with LELs of 10μM in all OPFRs in at least 1 endpoint, except for IDDP and TCPP. Moreover, in multiple endpoints the bioactive OPFRs were all equal to or more potent than BDE-47, which was phased-out due to concerns about its toxicity, emphasizing the importance of evaluating the toxicity of these replacement FRs, which were meant to lessen toxicity concerns. TBPH, BDE-135, TBB, TCEP, as well as the two negative control chemicals, were inactive in both worm types. The duplicate chemical, TPHP, was consistent between batches and thus the data is only shown once.

Additionally, 8 FRs in regenerating planarians (BDE-99, TBBPA, TCPP, EHDP, IPP, BPDP, TMPP and TPHP) and 6 FRs in full planarians (TCPP, EHDP, IPP, BPDP, TMPP and

TPHP) caused morphological and behavioral defects at day 12 sublethal concentrations where animal viability wasn't affected, suggesting specific non-systemic toxicity at these concentrations. The two BFRs BDE-99 and TBBPA didn't elicit sublethal effects in full planarians, due to increased sensitivity to lethality in the full worms. Similar increased sensitivity of full planarians to the lethal effects of some chemicals has been observed previously (Hagstrom et al., 2015; Zhang et al., 2018). Interestingly, TCPP affected almost all behavioral and morphological endpoints in both worm types in the absence of lethality. Scrunching, a musculature-driven gait used as an escape response to adverse stimuli (Cochet-Escartin et al., 2015), was the most sensitive endpoint with 7/10 and 7/11 FRs ,in regenerating and full planarians, respectively, causing scrunching defects at sublethal concentrations. Sublethal effects were also seen to a lesser extent on unstimulated behavior (regenerating planarians in TBBPA and full planarians in IPP) and eye regeneration (TBBPA). Hits due to hyper- (rather than hypo-) activity in unstimulated behavior or hits which were concentration-independent were not included in this analysis (Zhang et al., 2018). Together, these toxicological profiles suggest the importance of discerning sublethal toxicity from systematic toxicity.

By comparing effects in regenerating and full planarians, we found IDDP shows potential developmental selective defects in day 12 unstimulated behavior and phototaxis at 100μM, since it did not cause defects in these endpoints nor high lethality (<75%) in full planarians. TBBPA also caused a developmental selective defect in day 12 unstimulated behavior, since effects on this endpoint were seen at 10μM in regenerating but not full planarians. Of note, this concentration did induce lethality in 24% of full planarians at day 12 (see Supplemental File 1). However, this low level of lethality may suggest that any overt systemic toxicity at this concentration would not be potent enough to mask effects in other endpoints. Furthermore, 6 of

the 11 active FRs (TBBPA, FM550, TCPP, EHDP, BPDP, TMPP) caused eye regeneration defects, with TBBPA affecting regeneration at a sublethal concentration.

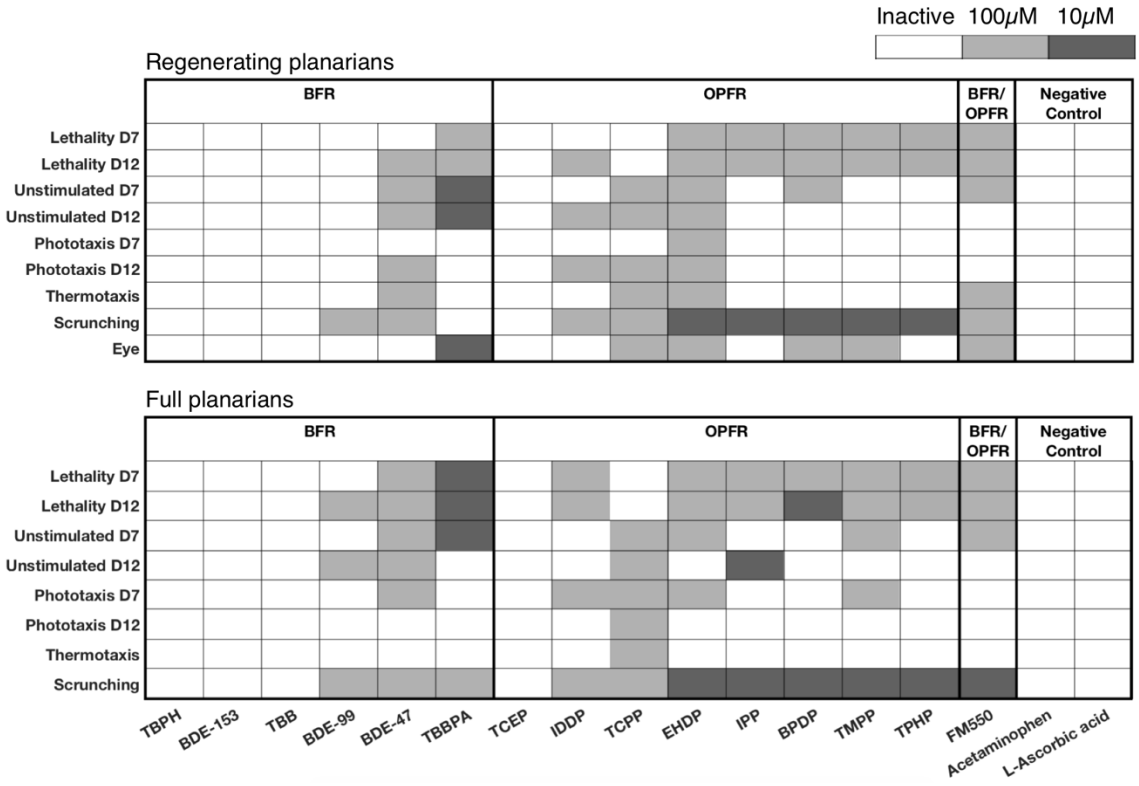


Figure 5.2. Overview of the planarian screening data of 15 flame retardants (FRs) and 2 negative controls. Heat maps of effects of brominated flame retardants (BFRs), organophosphorous flame retardants (OPFRs) and negative controls on regenerating planarians (Top) and full planarians (Bottom) for all endpoints with lowest effect level (LEL) color-coded. TPHP was screened as a duplicate, but only shown once here since the results were consistent. Note that BDE-153 was screened at a maximum concentration of 50 μM.

Concordance of active hits between planarians and published literature

Fourteen of the 15 FRs tested in this screen have been previously studied in nematode *C. elegans*, zebrafish, and *in vitro* cell-based assays with some FRs showing DT or DNT; thus, herein, we compare our planarian data with these published results (Behl et al., 2015; Behl et al., 2016; Jarema et al., 2015; Noyes et al., 2015; Noyes et al., 2015)(Table 5.2). The FM550 mixture was not screened in any of these previous studies and was thus excluded from this comparison. Two independent studies were compared for results in *C. elegans* focusing on larval development (Behl et al., 2015) and reproduction, and larval development and feeding (Behl et al., 2016). Three independent zebrafish studies focused on embryonic development (Behl et al., 2015), malformation, acute and developmental behaviors on 6 days-post-fertilization larvae (Jarema et al., 2015), versus developmental malformations and behaviors on 24 and 120 hours-post-fertilization embryos and larvae (Noyes et al., 2015). Different procedures were noted in different studies, which are necessary to bear in mind when performing direct comparisons. It should also be noted that of these studies some, including ours herein, evaluated toxicity potency using the nominal test concentrations (i.e. LEL) (Behl et al., 2016; Jarema et al., 2015; Noyes et al., 2015) while others used modeling approaches to calculate a point of departure (POD) (Behl et al., 2015).

Thirteen of the 14 FRs were previously found to be bioactive in at least one model. Eleven of 14 FRs were bioactive in more than one model and all 10 of the 11 bioactive FRs, with the exception of TCEP, were similarly bioactive in the planarian system. IPP is the only FR which was bioactive in all tested systems. TCEP was found to be inactive at the assayed concentrations in all tested systems except in the zebrafish screens by Noyes et al. (Noyes et al., 2015) and Jarema (Jarema et al., 2015). Notably, the zebrafish data from (Noyes et al., 2015)

shows increased sensitivity to 5 FRs (BDE-47, TBBPA, BPDP, IPP and TMPP) when compared to the other published data (Table 5.2). BDE-153, TBB , TBPH and TCPP were only tested in the zebrafish (Noyes et al., 2015) and planarian systems, where BDE-153 and TBPH were only found bioactive in the Noyes et al. zebrafish study, but not in planarians. TBB is the only FR which was found to be inactive in both zebrafish and planarians. Thus all systems showed high concordance with each other to suggest active chemicals.

Furthermore, we compared the sensitivity of our planarian system to these previously published results. The different approaches used to evaluate potency in LELs or PODs likely attribute to the variability among the reported effected concentrations. LELs are limited to the tested concentrations, and thus the respective POD would lie somewhere within the range of the LEL and the next lower tested concentration level. With this caveat in mind, 8 FRs (BDE-47, TBBPA, BPDP, EHDP, IPP, TMPP, TPHP, TCPP) were found to have overall LELs (from any endpoint) in the planarian system within an order of magnitude of effect levels (LEL or POD) in zebrafish, nematode and *in vitro* cell-based models (Figure 5.3, Table 5.2), suggesting comparable sensitivity.

Table 5.2. **Comparison of FR toxicity between regenerating planarians with different developing models**. Lowest effect level (LELs) or point-of-departure (POD) for each model are listed, in μM, and color-coded by order of magnitude. Dash (-) indicates that there is no toxicity detected at tested concentrations. An empty cell indicates that this chemical was not tested in this assay. Concentrations are in μM. The number in the brackets represents the LELs with the consideration of dose-independent and hyper-activity effect.

		BFRs						OPFRs							
Model	**Assay**	BDE-47	BDE-99	BDE-153	TBBPA	TBB	TBPH	BPDP	EHDP	IDDP	IPP	TCEP	TMPP	TPHP	TCPP
Planarian	Any endpoint (LEL)	100	100	-	10	-	-	10.05	10	100.5	10.05	-	10	9.95	100
Zebrafish	Embryonic development (POD)[a]	28.2			4.6			9.8	15.3	-	4.9	-	15.4	2	
	Acute and developmental behavioral (LEL)[b]	4			1.2			3.8 [1.2]	3.8 [1.2]	67.2 [12]	1.2	12	4	0.4	
	Any toxicity in developmenal malformations and behaviors (LEL)[d]	0.0064	0.64 [0.0064]	6.4 [0.0064]	0.0064	- [6.4E-4]	0.64 [6.4E-4]	0.064 [0.0064]	64 [0.0064]	6.4 [0.0064]	0.064	64 [0.0064]	0.0064	64 [0.0064]	64 [0.64]
Nematode	Larval development (POD)[a]	0.6			10.1			3.3	2.3	3.9	3.2	-	95.5	0.9	
	Any toxicity in reproduction, larval development and feeding (LEL)[c]	0.4	0.16		3.2			2.5	1.6	2.5	1.6	-	63	0.16	
In vitro	Mouse stem cell differentiation (POD)[a]	-			41			-	-	-	66.1	-	-	-	
	Human stem cell neuroprogenitor proliferation (POD)[a]	-			-			7.2	13.2	6.1	8.7	-	7.4	-	
	Rat neurite outgrowth (POD)[a]	-			-			14.9	-	-	8.7	-	-	-	
	Human stem cell neurite outgrowth (POD)[a]	5.7			12.2			4.1	6.9	4.7	13.9	-	-	15.9	
legend: magnitude of the LELs and PODs		<0.11μM			0.11μM - 1.1μM			1.1μM - 10.1μM				10.1μM - 100μM			

a. data from (Behl et al., 2015); b, data from (Jarema et al., 2015);c, data from (Behl et al., 2016); d, data from (Noyes et al., 2015)

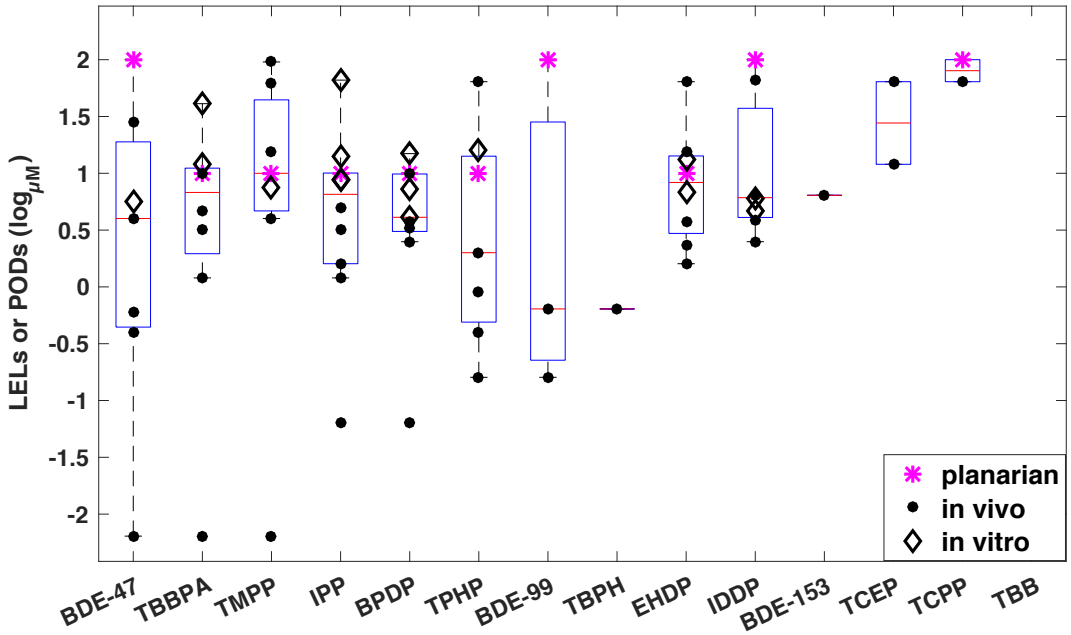


Figure 5.3. Comparison of toxicity of 10 FRs in regenerating planarians and other developing models. The box and whisker plot displays the LELs and PODs for different FRs measured in different models. The stars represent planarian LELs, and filled black dots represent LELs/PODs in zebrafish and nematode models (Behl et al., 2015; Behl et al., 2016; Jarema et al., 2015; Noyes et al., 2015), and black diamond represent PODs in vitro cell-based models (Behl et al., 2015) reported in these studies. The x-axis shows the FRs in the decreasing order of lowest reported toxicity.

Robustness of planarian FR screen.

We evaluated the robustness of the planarian screening platform by comparing the chemical hits and endpoint readouts detected from compiling different numbers of replicates from the two independent screens (FR screen 1 and FR screen 2) (Figure 5.4). Of the 18 screened chemicals, IPP and TMPP were provided by two different suppliers for the two screens (Table 5.1). Given potential unknown variability between the different chemical batches, IPP and TMPP were therefore excluded from the robustness evaluation. It should be noted that in the published zebrafish study (Noyes et al., 2015), significant readout differences on zebrafish were found in IPP from three different suppliers in the same screen.

Considering any assay endpoint, 10 of the 16 screened chemicals, including the duplicate TPHP, were deemed bioactive hits in both worm types in all sets of compiled data (3, 4, 5 or 6 replicates). Thus, the overall chemical hit-call did not differ regardless off the number of replicates used. Moreover, the overall LELs from any readout was found to be the same for almost all bioactive chemicals in 3/4/5 replicates versus 6 replicates. Only for BDE-47 was the overall LEL at any assay endpoint found to differ depending on the number of replicates analyzed. The overall LEL for full planarians exposed to BDE-47 was lower (i.e. more potent) when using 3 and 4 replicates compared to using 6 replicates. This resulted from significant effects being found at 10 μM BDE-47 in day 7/12 unstimulated behavior assay when analyzing 3 or 4 replicates but not 6, since the 3 replicates in FR screen 2 showed higher sensitivity in this assay.

Figure 5.4. Comparison of lowest effect levels (LELs) identified for each endpoint by compiling data from 3, 4, 5 or 6 replicates in regenerating and full (adult) planarians. Readouts, i.e. whether bioactivity was detected in a particular endpoint, were determined for each endpoint using 3, 4, or 5 replicates from the 2 flame retardant (FR) screens and compared with the readouts determined from 6 replicates. "-" indicates the chemical was inactive in that endpoint. Loss of a bioactive readout (deemed bioactive in 6 replicates but not 3, 4 or 5) are in dark grey. False positive readouts (deemed bioactive in 3, 4 or 5 replicates but not 6) are in yellow. Endpoint readouts which differed in sensitivity/potency (still bioactive in both screens but with different LELs) are in green. The overall LEL of each chemical is listed in a separate column. IPP and TMPP (in red text) were excluded from the robustness evaluation, since the chemical supplier differed between the two FR screens.

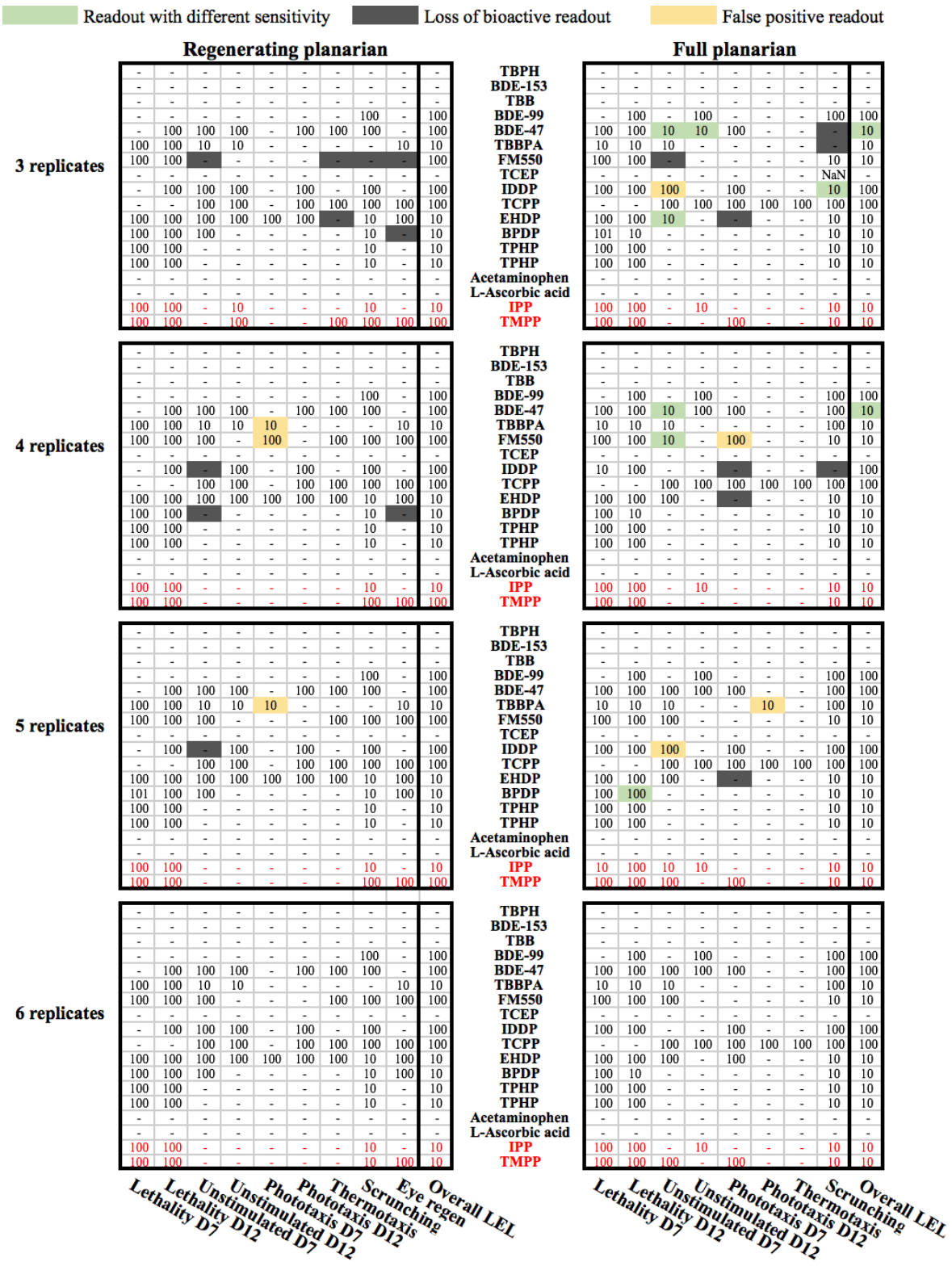

Readout with different sensitivity
Loss of bioactive readout
False positive readout
Regenerating planarian
Full planarian
3 replicates
4 replicates
5 replicates
6 replicates
TBPH
BDE-153
TBB
BDE-99
BDE-47
TBBPA
FM550
TCEP
IDDP
TCPP
EHDP
BPDP
TPHP
TPHP
Acetaminophen
L-Ascorbic acid
IPP
TMPP
Lethality D7
Lethality D12
Unstimulated D7
Unstimulated D12
Phototaxis D7
Phototaxis D12
Thermotaxis
Scrunching
Eye regen
Overall LEL

Table 5.3. Bioactivity concordance of readouts and sensitivity concordance of concordant bioactive readouts between the data using 3, 4 or 5 replicates compared with the data using 6 replicates. Data is shown for regenerating (R) and full (F, adult) planarians. IPP and TMPP were excluded from the analysis. Bioactivity concordance of readouts was determined as the number of readouts showing the same activity (bioactive or inactive) as using 6 replicates, out of the total number of readouts (9 for regenerating and 8 for full planarians) for all 16 chemicals. Sensitivity concordance of concordant bioactive readouts using 3/4/5 replicates was determined as the number of bioactive readouts with the same LEL as in 6 replicates, out of the total number of concordant bioactive readouts for all 16 chemicals in this set of compiled data.

	Bioactivity concordance of readouts		**Sensitivity concordance of bioactive concordant readouts**	
	R (144 readouts)	F (128 readouts)	R (concordant bioactive readouts)	F (concordant bioactive readouts)
3 replicates	96%	96%	100%	97%
4 replicates	97%	97%	100%	99%
5 replicates	99%	97%	100%	100%

We found that all chemical hit-calls (i.e. whether a chemical was toxic or not) determined by different numbers of replicates stay the same. Few differences in individual endpoints were found in the compiled data of 3,4 5 or 6 replicates, but none of the readout difference affect chemical hit-calls. To get more insight into the readouts, firstly, we compared the bioactivity of the readouts for each endpoint using the compiled data of 3, 4 or 5 replicates with the compiled data of all 6 replicates (Figure 5.4, Table 5.3). For the 16 chemicals, of the 144 readouts in regenerating planarians, the activity (either bioactive or inactive) determined with 6 replicates was found to be concordant for 138 (96%), 139 (97%), and 142 (99%) readouts using 3, 4, and 5 replicates, respectively. Similar readout bioactivity concordance rates were found in full planarians with the bioactivity of 123 (96%), 124 (97%), and 124 (97%) readouts in 3, 4, and 5 replicates being concordant with the bioactivity of the 128 readouts determined from 6 replicates. These differences were mostly due to loss of active readouts found in 3, 4 or 5 replicates, although a few false positive readouts were found. Generally, there were greater incidences of loss of active readouts as fewer replicates were compiled. This loss of bioactivity is mostly due to the smaller sample size limiting the ability to detect statistically significant effects. Particularly, for all readouts from 3 replicates and almost all readouts from 4 and 5 replicates (with the exception of the readouts for IDDP), all loss of active readouts occurred at concentrations causing significant lethality, thus there was not enough data from alive animals to yield a significant effect in the statistical test This suggests that some specific defects (e.g. scrunching defects) may be masked by overt systemic toxicity and lethality, especially in smaller sample sizes. For IDDP, differences in readout bioactivity were not due to low sample size but instead from inconsistency among the different replicates.

Furthermore, for the concordant bioactive readouts, we evaluated the sensitivity concordance by comparing the LELs determined from the different replicate sets (Table 5.2). In regenerating tails, all concordant bioactive readouts had the same LELs across all sets of compiled data. In adult planarians, 119/123 (97%), 123/124 (99%), and 124/124 (100%) of the concordant bioactive readouts showed the same LELs using 3, 4, and 5 replicates, respectively, compared with using 6 replicates. Therefore, together these data show that there was high reproducibility among the different replicates and that the majority of hits determined by using 3, 4 or 5 replicates were highly concordant with the hits in 6 replicates.

TPHP was screened as a duplicate in FR screen 2 (FR-specific library screen). The results of the duplicates were consistent in any set of compiled data, being bioactive in the same endpoints with the same LELs (Figure 5.4), which underlines the robustness and reproducibility of the planarian screening system.

In summary, through the comparison of the data using 3, 4, or 5 replicates with the data using 6 replicates, we found that the planarian system is robust and reproducible to identify bioactive chemicals. Three replicates were sufficient to identify all chemical hit-calls, providing the same list of bioactive chemicals as using 6 replicates. Moreover, different sets of replicates displayed very high concordance (>96%) of e readout bioactivity. Thirdly, looking into the sensitivity level (i.e. LELs) of the concordant bioactive readouts, again, all sets of replicates are highly concordant with the data using 6 replicates. All loss of active readouts was found at lethal concentrations in the data using 3 replicates, suggesting overt systemic toxicity which may be masking other readouts. Since all endpoints, except lethality, were quantified based on the data from living animals, the small sample size as a result of high lethality limits the power of statistical test to detect any significant effect. Moreover, lethality suggests overt systemic toxicity

at these concentration, the function-specific toxicity detected by other readouts is likely masked by the overt toxicity. Therefore, in this case, the sensitivity of assessing the bioactivity of the chemicals still remains. Given the overt toxicity at lethal concentrations, for more sensitive morphological and behavioral readouts, many labs mostly focus on the effects at sublethal concentrations, disregarding effects at lethal concentrations (Jarema et al., 2015; Truong et al., 2014). All together these data indicate that different number of replicates (from 3-6) yield very similar results, demonstrating the robustness of our current screening strategy using 3 replicates.

In addition, time and cost were taken into consideration for running additional replicates. Considering the time spent on plate preparation, screening, and data analysis for a set of 18 chemicals (our current maximum ability for a single unit of the platform to screen per day), it takes a minimum of 31, 39, 45 or 53 days to finish 3, 4, 5 or 6 replicates, respectively. Considering major expenses such as the multi-well plates, sealing films and DMSO solvent, it costs $306 to screen the minimum 3 replicates, with each additional replicate adding approximately $102. However, despite these increases in cost and time, more replicates yielded the same overall results as obtained with 3 replicates. Therefore, 3 replicates is the most economic choice with great robustness and reproducibility.

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References

Behl, M., Hsieh, J. H., Shafer, T. J., Mundy, W. R., Rice, J. R., Boyd, W. A., Freedman, J. H., Hunter, E. S., Jarema, K. A., Padilla, S. and Tice, R.R. (2015). Use of alternative assays to identify and prioritize organophosphorus flame retardants for potential developmental and neurotoxicity. *Neurotoxicol. Teratol.* **52**, 181–193.

Behl, M., Rice, J. R., Smith, M. V, Co, C. A., Bridge, M. F., Hsieh, J.-H., Freedman, J. H. and Boyd, W. A. (2016). Comparative toxicity of organophosphate flame retardants and polybrominated diphenyl ethers to Caenorhabditis elegans. *Toxicol. Sci.* **154**, 241–252.

Behl, M., Ryan, K., Hsieh, J.-H., Parham, F., Shapiro, A., Collins, B. J., Birnbaum, L. S., Bucher, J. R., Walker, N. J., Foster, P. M., et al. (2018). Screening for developmental neurotoxicity (DNT) at the National Toxicology Program: The future is now! *Toxicol. Sci.*

Bennett, D., Bellinger, D. C. and Birnbaum, L. S. (2016). Project TENDR: Targeting environmental neuro-developmental risks. The TENDR consensus statement. *Environ. Health Perspect.* **124**, A118-122.

Cochet-Escartin, O., Mickolajczk, K. J. and Collins, E.-M. S. (2015). Scrunching: a novel escape gait in planarians. *Phys. Biol.* **12**, 55001.

Costa, L. G. and Giordano, G. (2007). Developmental neurotoxicity of polybrominated diphenyl ether (PBDE) flame retardants. *Neurotoxicology* **28**, 1047–67.

Darnerud, P. O. (2003). Toxic effects of brominated flame retardants in man and in wildlife. *Environ. Int.* **29**, 841–853.

González-Alzaga, B., Lacasaña, M., Aguilar-Garduño, C., Rodríguez-Barranco, M., Ballester, F., Rebagliato, M. and Hernández, A. F. (2014). A systematic review of neurodevelopmental effects of prenatal and postnatal organophosphate pesticide exposure. *Toxicol. Lett.*

Hagstrom, D., Truong, L., Zhang, S., Tanguay, R. L. and Collins, E.-M. S. (2018) Comparative analysis of zebrafish and planarian model systems for developmental neurotoxicity screens using an 87-compound library Title. *Toxicol. Sci.*

Hagstrom, D., Cochet-Escartin, O., Zhang, S., Khuu, C. and Collins, E. M. S. (2015). Freshwater planarians as an alternative animal model for neurotoxicology. *Toxicol. Sci.* **147**, 270–285.

Hagstrom, D., Cochet-Escartin, O. and Collins, E.-M. S. (2016). Planarian brain regeneration as a model system for developmental neurotoxicology. *Regeneration* **3**, 65–77.

Hale, R. C., Alaee, M., Manchester-Neesvig, J. B., Stapleton, H. M. and Ikonomou, M. G. (2003). Polybrominated diphenyl ether flame retardants in the North American environment. *Environ. Int.* **29**, 771–779.

Herbstman, J. B., Sjödin, A., Kurzon, M., Lederman, S. A., Jones, R. S., Rauh, V., Needham, L. L., Tang, D., Niedzwiecki, M., Wang, R. Y. and Perera, F. (2010). Prenatal exposure to PBDEs and neurodevelopment. *Environ. Health Perspect.* **118**, 712–719.

Jarema, K. A., Hunter, D. L., Shaffer, R. M., Behl, M. and Padilla, S. (2015). Acute and developmental behavioral effects of flame retardants and related chemicals in zebrafish. *Neurotoxicol. Teratol.*

Meeker, J. D. and Stapleton, H. M. (2010). House dust concentrations of organophosphate flame retardants in relation to hormone levels and semen quality parameters. *Environ. Health Perspect.* **118**, 318–23.

Muñoz-Quezada, M. T., Lucero, B. A., Barr, D. B., Steenland, K., Levy, K., Ryan, P. B., Iglesias, V., Alvarado, S., Concha, C., Rojas, E., and Vega, C. (2013). Neurodevelopmental effects in children associated with exposure to organophosphate pesticides: A systematic review. *Neurotoxicology*.

Noyes, P. D., Haggard, D. E., Gonnerman, G. D. and Tanguay, R. L. (2015). Advanced morphological-behavioral test platform reveals neurodevelopmental defects in embryonic zebrafish exposed to comprehensive suite of halogenated and organophosphate flame retardants. *Toxicol. Sci.* **145**, 177–195.

Ricceri, L., Venerosi, A., Capone, F., Cometa, M. F., Lorenzini, P., Fortuna, S. and Calamandrei, G. (2006). Developmental neurotoxicity of organophosphorous pesticides: Fetal and neonatal exposure to chlorpyrifos alters sex-specific behaviors at adulthood in mice. *Toxicol. Sci.*

Salimi, M., Esrafili, A., Gholami, M., Jonidi Jafari, A., Rezaei Kalantary, R., Farzadkia, M., Kermani, M. and Sobhi, H. R. (2017). Contaminants of emerging concern: a review of new approach in AOP technologies. *Environ. Monit. Assess.* **189**, 414.

Slotkin, T. A., Levin, E. D. and Seidler, F. J. (2006). Comparative developmental neurotoxicity of organophosphate insecticides: Effects on brain development are separable from systemic toxicity. *Environ. Health Perspect.*

Stapleton, H. M., Kelly, S. M., Pei, R., Letcher, R. J. and Gunsch, C. (2009a). Metabolism of polybrominated diphenyl ethers (PBDEs) by human hepatocytes in vitro. *Environ. Health Perspect.* **117**, 197–202.

Stapleton, H. M., Klosterhaus, S., Eagle, S., Fuh, J., Meeker, J. D., Blum, A. and Webster, T. F. (2009b). Detection of Organophosphate Flame Retardants in Furniture Foam and U.S. House Dust. *Environ. Sci. Technol.* **43**, 7490–7495.

Stapleton, H. M., Eagle, S., Anthopolos, R., Wolkin, A. and Miranda, M. L. (2011). Associations between polybrominated diphenyl ether (PBDE) flame retardants, phenolic metabolites, and thyroid hormones during pregnancy. *Environ. Health Perspect.* **119**, 1454–9.

Stapleton, H. M., Sharma, S., Getzinger, G., Ferguson, P. L., Gabriel, M., Webster, T. F. and

Blum, A. (2012). Novel and high volume use flame retardants in US couches reflective of the 2005 PentaBDE phase out. *Environ. Sci. Technol.* **46**, 13432–13439.

Stapleton, H. M., Misenheimer, J., Hoffman, K. and Webster, T. F. (2014). Flame retardant associations between children's handwipes and house dust. *Chemosphere* **116**, 54–60.

Talsness, C. E. (2008). Overview of toxicological aspects of polybrominated diphenyl ethers: A flame-retardant additive in several consumer products. *Environ. Res.* **108**, 158–167.

Truong, L., Reif, D. M., St Mary, L., Geier, M. C., Truong, H. D. and Tanguay, R. L. (2014). Multidimensional in vivo hazard assessment using zebrafish. *Toxicol. Sci.* **137**, 212–33.

Tung, E. W. Y., Ahmed, S., Peshdary, V. and Atlas, E. (2017). Firemaster® 550 and its components isopropylated triphenyl phosphate and triphenyl phosphate enhance adipogenesis and transcriptional activity of peroxisome proliferator activated receptor (Pparγ) on the adipocyte protein 2 (aP2) promoter. *PLoS One* **12**, e0175855.

Van Der Veen, I. and De Boer, J. (2012). Phosphorus flame retardants: Properties, production, environmental occurrence, toxicity and analysis. *Chemosphere* **88**, 1119–1153.

Zhang, S., Hagstrom, D., Hayes, P., Graham, A. and Collins, E.-M. S. (2018). Multi-behavioral endpoint testing of an 87-chemical compound library in freshwater planarians. *Toxicol. Sci.*

Chapter 6: Comparative analysis of the mechanisms of organophosphorus pesticide developmental neurotoxicity in freshwater planarians

Abstract

Organophosphorus pesticides (OPs), are among the most prominent pesticides used in agriculture and kill insects by inhibiting acetylcholinesterase (AChE), leading to over-excitation of the cholinergic system. Growing evidence suggest that exposure to environmental concentrations of OPs during development may cause life-long neurological damage and behavioral disorders. However, it is debated whether these effects are due to AChE inhibition. Several alternative molecular targets have been suggested to be affected by OP exposure, including cytoskeletal proteins, regulators of endocannabinoid signaling, and oxidative stress, although the significance of these targets on functional adverse outcomes is unknown. Moreover, it is unclear whether different OPs, which can cause different adverse outcomes, act through the same mechanism(s). We therefore tested whether the distinct toxicological profiles induced by different OPs are due to differential effects on these alternative pathways by screening for effects on various morphological and behavioral readouts in asexual freshwater planarians. Using a custom robotic screening platform, a comparative screen was performed of 6 OPs (chlorpyrifos, chlorpyrifos oxon, dichlorvos, diazinon, malathion and parathion) and of compounds known to activate pathways suggested in the literature to be OP targets (cholinergic overstimulation, cytoskeletal depolymerization, endocannabinoid system activation, and oxidative stress). By comparing each toxicant's toxicological profile, we link specific mechanisms with their functional toxicological outcomes and determine the role these play in differential OP toxicity. Thus, we provide mechanistic insight into how different OPs can distinctly damage the developing brain and identify relevant molecular targets and the functional consequences of their disruption.

Introduction

Organophosphorus pesticides (OPs) are among the most agriculturally important and common pesticides used today (Atwood and Paisley-Jones, 2017; EUROSTAT, 2016). Because of their environmental abundance, it is alarming that growing evidence correlates chronic prenatal and infant exposure to subacute levels of OPs with life-long neurological damage and behavioral disorders (Burke et al., 2017; González-Alzaga et al., 2014; Muñoz-Quezada et al., 2013; Rauh et al., 2011; Shelton et al., 2014). Acute OP toxicity is due to inhibition of acetylcholinesterase (AChE) (Russom et al., 2014; Taylor, 2018), which is responsible for hydrolyzing the neurotransmitter acetylcholine (ACh). However, it is debated whether this is the predominant mechanism by which OPs cause developmental neurotoxicity (DNT), especially as some animal studies have observed OP-induced DNT in the absence of significant AChE inhibition (Mamczarz et al., 2016; Yang et al., 2008; Zarei et al., 2015) or found that the extent of AChE inhibition did not correlate with the presence of DNT, such as when comparing gender-selective effects of chlorpyrifos (CPF) exposure in rats (Dam et al., 2000). However, a direct link between disruption of non-AChE targets and neurodevelopmental defects and the extent that these and/or cholinergic mechanisms contribute to DNT has been difficult to ascertain. A multitude of potential alternative targets have been suggested to be affected by OP exposure, including the ACh receptors (AChRs), other esterases, and non-esterase, non-cholinergic targets such as cytoskeletal proteins (Burke et al., 2017; Carr et al., 2014; Flaskos, 2014; Pope, 1999; Pope et al., 2005; Slotkin et al., 2017). The impact of these effects is unclear, however, because few connections between molecular/cellular endpoints and brain function (behavioral) deficits have been made.

The majority of mechanistic OP research has been focused on the most abundant OP CPF.

Thus, it has been largely assumed that all OPs, due to their common action on AChE, act in the same way. However, comparative studies in rats have shown that different OPs damage the developing brain to varying extents, resulting in different adverse outcomes (Moser, 1995; Pope, 1999; Richendrfer and Creton, 2015; Slotkin et al., 2006), reinforcing the need to thoroughly evaluate individual OPs to better understand any potential compound-specific toxicity. Thus far, however, studies have been limited in scope to either 1-3 compounds at a time (Crumpton et al., 2000; Richendrfer and Creton, 2015; Slotkin et al., 2006; Slotkin et al., 2017) or only acute effects (Moser, 1995).

To fill this data gap, we utilized our automated high-throughput whole animal screening platform (Zhang et al., 2018) to perform a comparative screen of 6 OPs (CPF, chlorpyrifos oxon (CPFO), dichlorvos, diazinon, malathion, and parathion) in an asexual freshwater planarian, *Dugesia japonica.* These OPs were chosen because of their environmental abundance, differences in chemical structures, and known potency in planarians from our previous work quantifying the *in vitro* inhibition rates of the respective oxons (Hagstrom et al., 2017). CPF and its active oxon metabolite, CPFO, were both tested as it has been suggested that some of toxicity may occur from the parent form directly without bioactivation into CPFO (Crumpton et al., 2000). Planarians are an unique and apt system for developmental neurotoxicology, as development can be induced by amputation, wherein the tail piece will regenerate a new brain within 12 days (Hagstrom et al., 2016). As full and amputated regenerating planarians are of similar size, adult and regenerating animals can be tested in parallel with the same assays, providing the unique opportunity to directly identify effects specific to development. Planarian neuro-regeneration shares fundamental processes with vertebrate neurodevelopment. Moreover, the planarian central nervous system, while morphologically simple, has considerable cellular

and functional complexity (Cebrià, 2007; Ross et al., 2017). Planarians and mammals share key neurotransmitters (Ribeiro et al., 2005), including ACh, which has been shown to regulate motor activity in *D. japonica* (Nishimura et al., 2010). Moreover, our previous work identified 2 putative genes responsible for cholinesterase function in *D. japonica* which were sensitive to OP inhibition and whose knockdown recapitulated some phenotypes of subacute OP exposure (Hagstrom et al., 2017; Hagstrom et al., 2018). Lastly, planarians have a variety of different quantifiable behaviors which can be assayed to assess neuronal functions. Importantly, many of these behaviors have been shown to be coordinated by distinct neuronal subpopulations (Birkholz and Beane, 2017; Inoue et al., 2014; Nishimura et al., 2010) allowing us to link functional adverse outcomes with distinct cellular effects.

To delineate the molecular mechanisms underlying OP toxicity, we compared the toxicological profiles of 6 OPs to chemicals with known modes of action. These included cholinergic activators, such as carbamate AChE inhibitors (aldicarb and physostigmine) and nicotinic and muscarinic AChR agonists (nicotine/anatoxin-a and muscarine/bethanechol, respectively). We also tested several alternative targets suggested in the literature to be affected by OPs. First, as cytoskeletal proteins such as actin and tubulin have been suggested to be direct targets of OPs (Flaskos, 2012; Flaskos, 2014; Jiang et al., 2010; Zarei et al., 2015), we tested the cytoskeletal depolymerization drugs, cytochalasin D and colchicine. Second, fatty acid amide hydrolase (FAAH) has been shown to be inhibited by CPF leading to accumulation of the endocannabinoid anandamide and subsequent activation of the CB-1 receptor (Carr et al., 2014; Casida and Quistad, 2004; Liu et al., 2013). Thus, we characterized the toxicological effects of anandamide and the CB-1 receptor agonist WIN 55 212-2, which has been shown previously to affect planarian behavior (Buttarelli et al., 2002). Lastly, to test the effects of oxidative stress, a

common mechanism of toxicity also suggested to play a role in OP DNT (Crumpton et al., 2000; Singh et al., 2018), we evaluated the effects of rotenone and L-buthionine sulfoxime. Using this comparative approach, we find DNT induced by different OPs falls into one of three major groupings: 1) toxicity mainly due to cholinergic overstimulation (DDVP, malathion), 2) toxicity mainly due to endocannabinoid stimulation (CPF, CPFO, and parathion), and 3) other, non-classifiable (diazinon). Moreover, the endpoints affected by the OPs differed between adult and regenerating planarians, reinforcing the unique utility of the planarian system to identify development-specific toxicity. Together, these results provide new insight into the mechanisms of compound-specific OP DNT and provide new evidence for the important role non-cholinergic targets, specifically the endocannabinoid system, can play in specific toxic outcomes.

Material and methods

Test animals

Freshwater planarians of the species *Dugesia japonica,* originally obtained from Shanghai University, China and cultivated in our lab for > 5 years, were used for all experiments. Planarians were stored in 1x Instant Ocean (IO, Blacksburg, VA) in Tupperware containers and kept at 20°C in a Panasonic refrigerated incubator in the dark. The animals were fed organic freeze-dried chicken liver (either Mama Dog's or Brave Beagle, both from Amazon, Seattle , WA) once a week and their aquatic environment cleaned twice a week (Dunkel et al., 2011). For all experiments, only fully regenerated worms which had not been fed within one week and which were found gliding normally in the container were used. Worms were manually selected to fall within a certain range of sizes, with larger planarians used for amputation/regeneration experiments, such that the final sizes of adult and regenerating tails were similar. To induce development/regeneration, intact planarians were amputated on day 1 by cutting posterior to the auricles and anterior to the pharynx with an ethanol-sterilized razor blade. Exposure began within 3 hours of amputation. Of note, for animals which underwent fission during the course of the screen, only the head piece was considered in all analyses, as this would represent the first regenerated brain (Zhang et al., 2018).

Chemical preparation

Table 6.1 lists the chemicals used in this study. Two negative control chemicals, D-glucitol and L-ascorbic acid, which we have previously shown do not affect planarian behavior or morphology (Zhang et al., 2018), were also screened. Stock solutions were prepared in 100% dimethyl sulfoxide (DMSO, Sigma-Aldrich, Saint Louis, MO), with the exception of anatoxin-a, muscarine, and L-buthionine sulfoxime (BSO), which were prepared in water due to low

solubility in DMSO. All stock solutions were stored at -20ºC. For each chemical, 5 concentrations were tested. The highest concentrations were chosen, based on preliminary tests, to be at the threshold to cause lethality or overt systemic toxicity or the highest soluble concentration. The remaining concentrations are serial half-log dilutions (Table 6.1).

The set of 20 compounds was separated into 2 "Chemical Sets" of 10 chemicals, such that one chemical from each "class" (see Table 6.1) was tested in each Chemical Set. Chemicals in the same Chemical Set were tested on the same day, i.e. the same experiment. For the majority of the chemicals, 0.5% DMSO was used as solvent control, which we have previously shown has no effects on planarian morphology or behavior (Hagstrom et al., 2015). For chemicals prepared in water (anatoxin-a, muscarine, BSO), IO water was used as a control. Chemical stock plates were prepared in 96-well plates (Genesee Scientific, San Diego, CA) by adding 200X stock solutions in DMSO or water from the highest tested concentration to one well of the plate. Half-log serial dilutions were then made in DMSO or water with a multi-pipettor. The control well contained DMSO or IO water only. Stock plates were sealed and stored at -20 ºC.

Table 6.1. Chemicals tested in this screen.

Chemical Name	CAS	Class/mode of action	Concentrations tested (µM)	Supplier	Purity (%)	Chemical Set
Chlorpyrifos (CPF)	2921-88-2	OP	31.6, 10, 3.16, 1, 0.316	Sigma-Aldrich	100	1
Chlorpyrifos oxon (CPFO)	5598-15-2	OP	3.16 ,1, 0.316 , 0.1, 0.0316	Chem Service	98.8	1
Diazinon	333-41-5	OP	31.6, 10, 3.16, 1, 0.316	Sigma-Aldrich	98	1
Dichlorvos (DDVP)	62-73-7	OP	3.16, 1, 0.316, 0.1, 0.0316	Sigma-Aldrich	98	2
Malathion	121-75-5	OP	31.6, 10, 3.16, 1, 0.316	MP Biomedicals	96	2
Parathion	56-38-2	OP	31.6, 10, 3.16, 1, 0.316	Sigma-Aldrich	100	2
Aldicarb	116-06-3	Carbamate AChE inhibitor	316, 100, 31.6, 10, 3.16	Sigma-Aldrich	98	2
Physostigmine	57-47-6	Carbamate AChE inhibitor	10, 3.16, 1, 0.316, 0.1	Sigma-Aldrich	99	1
Anatoxin-A	64285-06-9	Nicotinic AChR agonist	100, 31.6, 10, 3.16, 1	Abcam	98	1
Nicotine	54-11-5	Nicotinic AChR agonist	1000, 316, 100, 31.6, 10	Sigma-Aldrich	98	2
Bethanechol	590-63-6	Muscarinic AChR agonist	316u, 100, 31.6, 10, 3.16	TCI America	98	2
Muscarine	2936-25-6	Muscarinic AChR agonist	100, 31.6, 10, 3.16, 1	Sigma-Aldrich	98	1
Colchicine	64-86-8	disrupts microtubule polymerization	316, 100, 31.6, 10, 3.16	Acros Organics	97	2
Cytochalasin D	22144-77-0	disrupts actin polymerization	31.6, 10, 3.16, 1, 0.316	MP Biomedicals	99	1
Anandamide	94421-68-8	Endocannabinoid	100, 31.6, 10, 3.16, 1	Sigma-Aldrich	97	1
WIN 55 212-2	131543-23-2	CB-1 receptor agonist	10, 3.16, 1, 0.316, 0.1	Sigma-Aldrich	98	2
L-buthionine sulfoxime	83730-53-4	induces oxidative stress	10000, 3160, 1000, 316, 100	Sigma-Aldrich	97	2
Rotenone	83-79-4	induces oxidative stress	0.316, 0.1, 0.0316, 0.010,0.00316	Sigma-Aldrich	100	1
L-ascorbic acid	50-81-7	Negative control	100, 31.6, 10, 3.16, 1	Alfa Aesar	99	1
D-glucitol	50-70-4	Negative control	100, 31.6, 10, 3.16, 1	Sigma-Aldrich	99	2

Screening plate setup

Each 48-well screening plate (Genesee Scientific) assayed 8 planarians in the solvent control (0.5% DMSO or IO water), and 8 planarians each per concentration of chemical (5 test concentrations per plate). Experiments were performed in triplicate (independent experiments performed on different days). The orientation of the concentrations in the plate was shifted down 2 rows in each replicate to control for edge effects (Zhang et al., 2018). For each chemical and experiment, one plate containing full (intact) planarians and one plate containing regenerating tails (2 plates total) were assayed.

On the day of plate set-up, the appropriate 200X chemical stock plate was thawed at room temperature for approximately 30 minutes. The 200X stocks were then diluted 20X in IO water to create 10X stock plates. These plates were mixed by rotation on an orbital shaker for approximately 10 minutes before use.

Screening plates were prepared as described in (Zhang et al., 2018) with one full planarian or amputated tail piece in each well of a 48-well plate containing 200µl of the nominal concentration of test solution and sealed with ThermalSeal RTS seals (Excel Scientific, Victorville, CA). The plates were stored, without their lids, in stacks in the dark at room temperature when not being screened. Since we previously found that fissioning worms produced challenges in our automated data analysis pipeline (Zhang et al., 2018) and because planarian fission is suppressed when disturbed (Malinowski et al., 2017), the plates were gently agitated by hand once every day when not being screened to discourage fission. Prepared plates were only moved to the screening platform when screened at day 7 and day 12.

Screening platform

We have further expanded the custom-built planarian screening platform described in (Zhang et al., 2018). Briefly, the platform consists of a commercial robotic microplate handler (Hudson Robotics, Springfield Township, NJ), two custom-built imaging systems and multiple assay stations. The imaging systems, assay stations and plate handler were controlled automatically by the computer. In addition to the assays performed in (Zhang et al., 2018), we have expanded the platform in the following ways (described in detail below): 1) expansion of the phototaxis assay to test both blue and green light stimuli, 2) modification of the scrunching assay to capture differences in the timing of reaction, and 3) addition of an automated "stickiness" assay. Moreover, analysis of the morphology/regeneration assay was expanded to also detect body shape changes.

In the expanded phototaxis assay, we replaced the previously used blue LED lights (Zhang et al., 2018) with RGB lights (DAYBETTER, Shenzhen, China) to test reactions to both green and blue light stimuli, building upon a previous study that showed that planarians detect blue, but not green, light with pigment in the skin in addition to their photoreceptors in the eyes (Birkholz and Beane, 2017; Paskin et al., 2014). Therefore, using the separate green and blue light stimuli allows us to discern between effects specific to the photoreceptors (green light) versus effects on extraocular perception through the skin. The expanded assay was performed in the following steps. First, to lower the variability of the animals' background activity, the plate was placed onto the phototaxis station 4 minutes prior to the assay, allowing the planarians in the plate to acclimate. After 4 minutes, the plate was imaged for 5 minutes: 1-min red light acclimation (1st dark cycle), 1-min green light stimulation (light cycle), 2-min red light acclimation (2nd dark cycle), 1-min blue light stimulation (light cycle). Of note, the second dark

cycle was 2 minutes to allow the planarians to acclimate and settle before the blue light stimulation, but only the activity in the last minute in the 2nd dark cycle was analyzed. The average speed in each 1-min dark and light cycle was quantified as in (Zhang et al., 2018). However, the phototactic response was quantified by calculating the difference of the average speed in each light cycle to that in the preceding dark cycle:

$$\Delta_{green\ light} = average\ speed_{green\ light\ cycle} - average\ speed_{1st\ dark\ cycle}$$

$$\Delta_{blue\ light} = average\ speed_{blue\ light\ cycle} - average\ speed_{2nd\ dark\ cycle}$$

Dead planarians were discarded from the analysis.

A new assay, termed "stickiness assay" since it quantifies the worm's tendency to stick to the substrate (Hagstrom et al., 2018; Malinowski et al., 2017), was added to the screening platform. We used a microplate orbital shaker (Big Bear Automation, Santa Clara, CA) to create controlled water flow to unstick the planarians from the bottom of the plate well. Different rotation speeds for full planarians and regenerating tails at day 7 and 12 were chosen based on preliminary testing to achieve a reproducible majority fraction of wild-type planarians to unstick. This intermediate unsticking capacity was chosen to be able to detect both an increase or decrease in planarian "stickiness". Day 7 for regenerating tails was observed as the relatively stickiest time-point, potentially due to locally increased secretion of mucus since the worms are less motile during regeneration. At day 7, the full planarian plates were shaken for 3 seconds at 552 revolutions per minute (rpm) and the tail plates for 3 seconds at 1017 rpm. Based on preliminary testing, full planarians kept in the screening plates for 12 days require greater water flow to be unstuck. Therefore at day 12, both full and tail plates, which are now more like adult animals (Hagstrom et al., 2015), were shaken for 3 seconds at 665 rpm. The plate was imaged from above by a USB3 camera (FLIR Systems Inc., Wilsonville, OR) mounted on a ring stand

and imaged at 8 frames per second (fps). Each worm was manually scored as either "unstuck" (defined as being displaced by the water flow and floating in the well) or "stuck" (defined as worms which did not float during the whole plate shaking session). The fraction of unstuck planarians was calculated as:

$$Fraction\ \ of\ unstuck\ planarians = \frac{the\ number\ of\ unstuck\ planarians}{the\ number\ of\ alive\ planarians}$$

In the morphology assay, different body shapes were classified for each alive planarian, including normal body shape, general sickness (lesions), contraction and ruffling, curled up or C-shape, corkscrew-like, head regression, pharynx extrusion, and tail anchored with head flailing around (Figure 6.1). Of note, one animal could be classified as having multiple body shapes, for example, C-shape and pharynx extrusion.

All assays were performed in the following order, whereby the notation in brackets indicates on which day(s) the assay was performed: phototaxis (d7/d12), unstimulated locomotion (d7/d12), lethality/regeneration (d7/d12), stickiness assay (d7/d12), thermotaxis (d12), and scrunching (d12). Data analysis was performed blinded by one investigator with no chemical information provided.

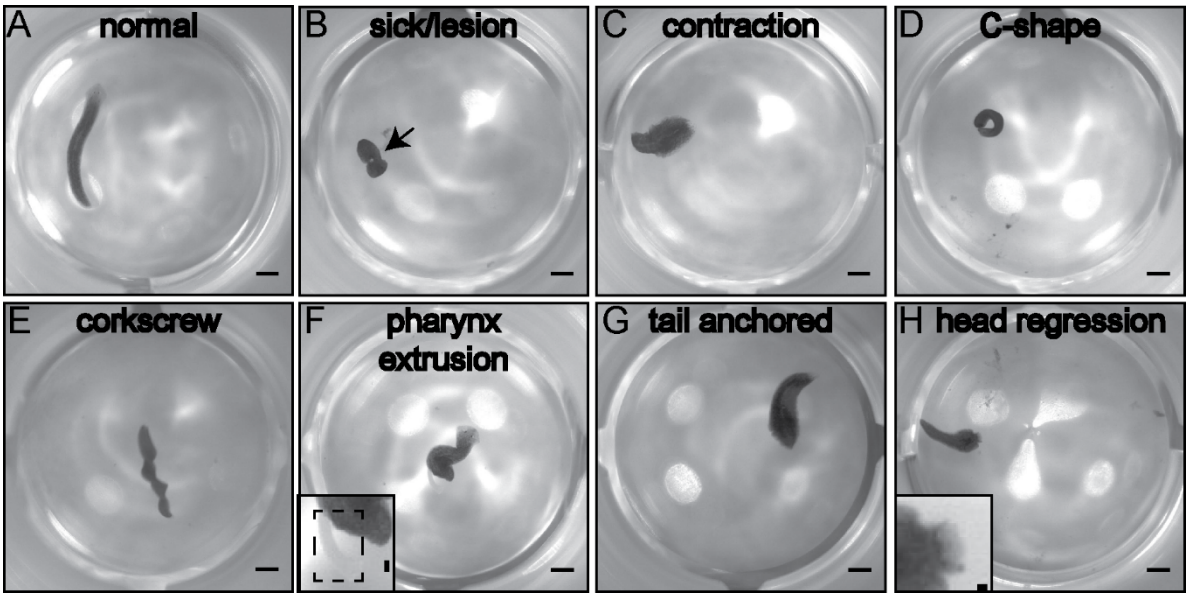

Figure 6.1. Body shape classifications in the morphology assay. High-resolution imaging was used to classify the body shape of the exposed planarians. Classifications of body shape included: (A) normal, (B) general sickness, with or without lesions (shown with an arrow), (C) contraction, ruffling of periphery, (D) curled or C-shape, (E) corkscrew-like hyperkinesia, (F) pharynx extrusion, inset shows close-up of the pharynx (shown in a dashed box) which is extended outside of the body, (G) tail anchoring while the head is freely moving, and (H) head regression, inset shows a close-up of the head which has disintegrated. Main scale bars are 1mm. Inset scale bars are 0.1mm.

Statistical testing

Statistical testing was performed on the compiled data from the triplicate runs. For all endpoints comparisons were made between the test population and the internal set of controls for that chemical. For lethality, eye regeneration, body shape morphology, stickiness, phototaxis and scrunching endpoints, a one-tailed Fisher's exact test was used. For thermotaxis and unstimulated behavioral endpoints, Tukey's interquartile test was first used to remove any outliers, with at most 5% of the data removed. A non-parametric one-tailed Mann Whitney U-test was used to determine significant effects in thermotaxis. For unstimulated behavior endpoints (speed and fraction of time resting), Lilliefors test was first used to test the normality of the samples. Thus, we performed either a parametric two-tailed t-test or a nonparametric two-tailed Mann-Whitney U-test depending on whether the sample distributions were normal or not, respectively. A sample was determined to be defective in unstimulated behavior if there was a significant difference in either speed or fraction of time resting compared with the controls. For all endpoints, significance was determined by a p-value less than 0.05. Biological relevancy cutoffs were used to remove effects within the assay-specific variability of all controls, as in (Zhang et al., 2018). Moreover, inconsistencies between the triplicate runs, wherein a single plate was responsible for designating a "hit", were flagged and excluded as hits. The lowest observed effect level (LOEL) was determined as the lowest tested concentration which showed a significant effect (statistically and biologically). If dose response for a particular endpoint was found to be non-monotonic, the lowest significant concentration is reported and flagged (asterisks in Figures 6.2 and 6.3). All statistical analyses were performed in MATLAB.

Results and Discussion

Exposure to the 6 OPs elicits different types of DNT

Using our multi-dimensional planarian screening platform, we characterized the toxicological effects of 6 OPs (CPF, CPFO, DDVP, diazinon, malathion, and parathion) on various morphological and behavioral endpoints. For our comparative analysis, we will focus on the results obtained in regenerating planarians (Figure 6.2), as this would be the most relevant for understanding developmental neurotoxicity (DNT) elicited by these compounds.

In agreement with other studies (Moser, 1995; Pope, 1999; Richendrfer and Creton, 2015; Slotkin et al., 2006), and our previous work with CPF and DDVP (Hagstrom et al., 2015), we found that exposure to the different OPs elicited a wide and variable range of phenotypes. The most widespread effects were seen with CPF and CPFO which caused effects in lethality, body shape, eye regeneration, increased stickiness, unstimulated behavior and blue light phototaxis. The only differences observed were that CPF, but not CPFO, caused defects in day 12 unstimulated behavior and blue light phototaxis, despite both compounds causing such defects at day 7. However, these day 12 differential effects are only seen at a lethal concentration of CPF, and thus may represent further manifestations of the systemic toxicity observed at this concentration. CPFO tended to have lightly more potency in regards to endpoints affected at sublethal concentrations (i.e. concentrations not deemed active in the lethality assay), as all affected morphological and behavioral phenotypes, except for body shape at day 12, occurred below the threshold for lethality. On the other hand, only day 7 body shape, increased stickiness (day 7/12), and unstimulated behavior (day 7) were affected at sublethal concentrations of CPF. For both CPF and CPFO, the most sensitive endpoint (i.e. endpoint affected at the lowest LOEL) is day 7 body shape (Table 6.1). Although some studies have suggested that CPF may cause

toxicity independently of its bioactivation into CPFO (Crumpton et al., 2000), our data suggests that exposure to either the parent or oxon form results in very similar adverse outcomes in regenerating planarians.

Many, but not all, of the endpoints affected by CPF/CPFO were also affected by malathion and DDVP. Both malathion and DDVP exposure caused effects at sublethal concentrations on body shape (day 7/12), increased stickiness (day 7/12), unstimulated behavior (day 7/12), and thermotaxis. All of these endpoints, with the exception of thermotaxis, were also affected by CPF/CPO exposure. However, several of these effects were only induced at lethal concentrations of CPF or CPFO, but were not concomitant with lethality in DDVP and malathion, suggesting that DDVP and malathion may be more potent at eliciting sublethal non-systemic toxicity. In addition to these shared phenotypes, malathion also caused defects in eye regeneration. Similarly to CPF and CPFO, the most sensitive endpoint to malathion exposure was day 7 body shape (Table 6.2). Unlike malathion, but similar to CPF, DDVP also caused defects in blue light phototaxis (day 7/12); however, the most sensitive endpoint was increased stickiness at day 7 with effects as low are 0.03 μM (the lowest tested concentration).

Conversely, parathion and diazinon affected fewer endpoints than the other 4 tested OPs. Parathion toxicity was mostly observed at a lethal concentration (32 μM). Of note, morphological and behavioral effects at this concentration, including body shape, blue light phototaxis, and eye regeneration, were only quantified at day 7 because all planarians were dead by day 12. The only effect observed at a sublethal concentration of parathion (3.16 μM) was hyperactivity in the day 12 blue light phototaxis assay. This effect was not concentration-dependent, but because it was shared among different replicates and in both regenerating and adult planarians, it is unlikely to be an artifact. Together, this toxicological profile suggests that

parathion toxicity is a result of systemic toxicity and not any specific DNT. This is in agreement with previous studies showing that in zebrafish and neonatal rats, parathion induces lethality before producing the significant levels of AChE inhibition or neurodevelopmental effects seen with CPF (Slotkin et al., 2006; Yen et al., 2011). Unlike all the other OPs tested, diazinon only caused a specific effect on increased planarian stickiness at both day 7 and 12 at as low as 3.2 μM, and in the absence of lethality. The absence of systemic toxicity for diazinon at the tested concentration range was surprising to us, since the oxon form of diazinon (diazinon oxon) was found to be the most potent of all OPs tested here at inhibiting DjChE activity *in vitro* (Hagstrom et al., 2017). Thus, we would predict that diazinon has the greatest potential to produce cholinergic shock leading to lethality. However, the lack of systemic toxicity or lethality at up to 31.6 μM, which was sufficient to cause lethality for CPF and parathion, suggests alternative mechanisms may be involved as well. It should be noted that CPF, parathion, and diazinon are all diethyl organothiophosphates with similar structures and pharmacokinetic properties, thus differences in uptake and metabolism are likely negligible. However, direct measurements of AChE activity at these concentrations should be performed to confirm this hypothesis.

Thus, in summary, although the precise endpoints affected by the OPs did vary considerably and no one endpoint was affected by all OPs, some endpoints were shared by the majority of the OPs. 5/6 OPs caused defects in body shape (day 7) and increased stickiness (day 7 and 12). These two endpoints also comprise the most sensitive endpoints in all the OPs tested and thus are sensitive predictors of OP toxicity in regenerating planarians. Planarian body shape has been previously shown to be a sensitive and characteristic readout for pharmacological manipulation of neurotransmitter systems. For example, cholinergic stimulation has been shown to induce “fixed postures” akin to our contraction classification, whereas dopaminergic

stimulation produces planarian hyperkinesia (Buttarelli et al., 2008). In agreement with this, the most common body shape classifications observed in OP-exposed worms were contraction and C-shapes, thus suggesting cholinergic stimulation. On the other hand, increased secretions (including bronchial, lacrimal, salivary, sweat, and intestinal secretions) are a major hallmark of acute cholinergic toxicity due to stimulation of muscarinic AChRs (Pope et al., 2005; Taylor, 2018). We have previously shown that increased planarian stickiness is associated with increased mucus secretion (Malinowski et al., 2017). Thus, the shared effects of the OPs on planarian stickiness may also be a result of the OPs shared action on AChE inhibition.

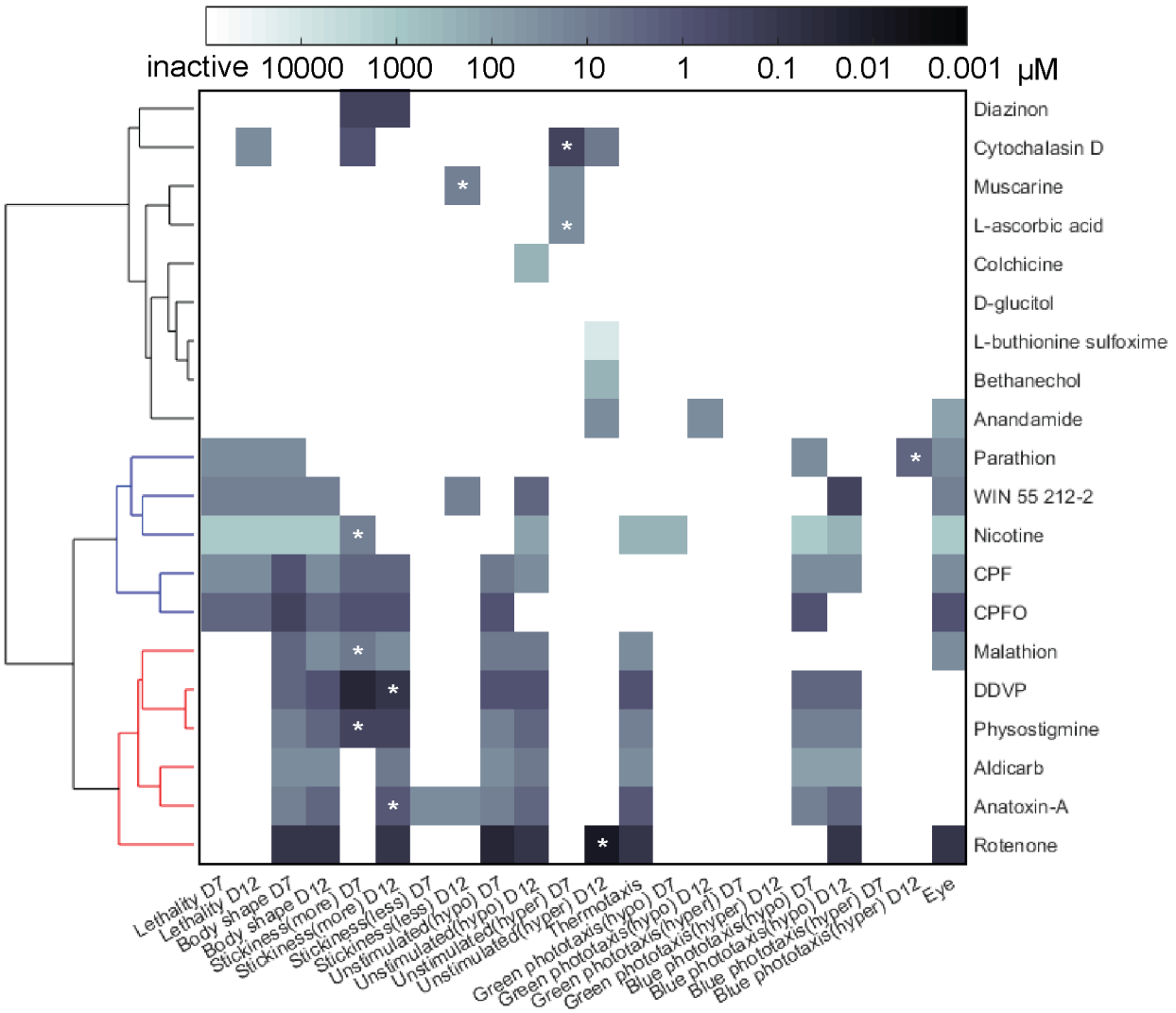

Figure 6.2. Regenerating planarian toxicological profiles. Heat map of endpoints affected in regenerating planarians for each chemical with LOEL color-coded. The chemicals' hits were clustered using Ward's method by calculating Euclidean distance between LOELs. LOELs defined by non-monotonic dose responses are marked with *. Clusters were manually color-coded for ease of comparison.

Table 6.2. Most sensitive endpoints affected by each chemical in regenerating planarians. List of endpoints affected at the overall lowest observed effect level (LOEL) for each compound. If a response is non-monotonic, the parameters are marked with (*) and the next highest concentration with a concentration-dependent response is also listed.

	Overall LOEL (uM)	**Most sensitive endpoint**
Chlorpyrifos (CPF)	1	body shape (day 7)
Chlorpyrifos oxon (CPFO)	0.32	body shape (day 7)
Diazinon	0.32	↑ stickiness (day 7/12)
Dichlorvos (DDVP)	0.032	↑ stickiness (day 7)
Malathion	3.2	body shape (day 7)
Parathion	3.2*; 32	↑blue light phototaxis (day 7); lethality (day 7/12), body shape (day 7), ↓blue light phototaxis (day 7), eye regeneration
Aldicarb	10	↑ stickiness (day 12), ↓unstimulated behavior (day 12)
Physostigmine	0.32	↑ stickiness (day 7*/12)
Anatoxin-A	1	↑ stickiness day 12*, thermotaxis
Nicotine	10*; 316	↑ stickiness (day 7)*; thermotaxis, ↓ green phototaxis (day 7), ↓ blue phototaxis (day 12)
Bethanechol	316	↑ unstimulated behavior (day 12)
Muscarine	10*; 32	↓ stickiness (day 12)*; ↑ unstimulated (day 7)
Colchicine	316	↓unstimulated behavior (day 12)
Cytochalasin D	0.32*; 1	↑ unstimulated behavior (day 7)*; ↑ stickiness (day 7)
Anandamide	32	↓unstimulated behavior (day 12), ↓ green light phototaxis (day 12)
WIN 55 212-2	0.32	↓ blue light phototaxis (day 12)
L-buthionine sulfoxime	10000	↑unstimulated behavior (day 12)
Rotenone	0.01*; 0.032	↑ unstimulated behavior (day 12)*; ↓ unstimulated behavior (day 7)
L-ascorbic acid	32*	↑ unstimulated behavior (day 7)*
D-glucitol	inactive	N/A

Comparison of OP toxicity with known effectors of mechanistic pathways

The phenotypes shared among the OPs suggest that some toxicity is likely due to cholinergic stimulation. To dissect the underlying mechanisms of OP DNT, comprising both shared and compound-specific effects, we compared the toxicological profiles of the 6 tested OPs with that of chemicals known to target pathways suggested to be affected by OPs, including cholinergic overstimulation, endocannabinoid system stimulation, oxidative stress and cytoskeletal depolymerization. The clinical manifestations of cholinergic shock are typically a mix of effects on downstream nicotinic and muscarinic AChRs (Taylor, 2018). Thus, to dissect whether some planarian toxicology profiles are affected by one or both pathways, we characterized the phenotypes of animals treated with agonists for either the nicotinic or muscarinic AChRs as well as carbamate AChE inhibitors, which would theoretically cause downstream effects in both. Ward's method of clustering was used to determine whether the toxicological profiles produced by these mechanistic control chemicals would group with any of the tested OPs. Focusing on the results in regenerating planarians, we found the toxicological profiles of the 20 chemicals (6 OPs, 12 mechanistic controls, and 2 negative controls) clustered into 3 main groups in regenerating planarians, denoted in red, blue, and black in Figure 6.2.

The red cluster consists of malathion, DDVP, physostigmine, aldicarb, anatoxin-A, and rotenone. Containing 3 cholinergic stimulators (2 carbamate AChE inhibitors and 1 nicotinic AChR agonist), this grouping may represent common effects due to cholinergic stimulation. All chemicals in this cluster caused effects in body shape (day 7/12), increased stickiness (day 12), unstimulated behavior (day 7/12), and thermotaxis. The majority of the chemicals (with the exception of malathion and rotenone for day 7, and only malathion for day 12) also caused defects in blue light phototaxis. Although rotenone, a pesticide which disrupts mitochondrial

function and thus induces oxidative stress, was also part of this cluster, it was not as closely linked as the other 5 chemicals. Just as in the OPs overall, all cholinergic stimulators in this cluster (physostigmine, aldicarb, and anatoxin-a) shared increased stickiness as one of their most sensitive endpoints (Table 6.2), further substantiating this endpoint as a readout of cholinergic overstimulation.

Another nicotinic AChR agonist, nicotine, also affected many of the same endpoints found in the red cluster, including body shape morphology (day 7/12), unstimulated behavior (days 12), thermotaxis, and blue light phototaxis (day 7/12). However, unlike the other cholinergic stimulators, nicotine only caused increased stickiness at day 7, but not day 12. In addition, defects were also observed in green light phototaxis at day 7 and eye regeneration. Moreover, some of these effects were observed at a lethal concentration (1 mM). Thus, the toxicological profile of nicotine differed enough to create a separate cluster (blue in Figure 6.2) from the other cholinergic stimulators. This cluster, also contained parathion, CPF, CPFO, and the CB-1 receptor agonist, WIN 55 212-2, and was mainly characterized by additional defects in lethality and eye regeneration, while losing some of the effects on day 7 stickiness, unstimulated behavior and thermotaxis observed in the red cholinergic cluster. It may be surprising that nicotine did not group more closely with the carbamate inhibitors or the other nicotinic agonist anatoxin-A. However, interestingly, studies have suggested there may be extensive cross-talk between nicotine and the endocannabinoid system (Gamaleddin et al., 2015), which may explain the similar effects seen with WIN 55 212-2 and nicotine.

Lastly, the remaining chemicals (diazinon, cytochalasin D, muscarine, L-ascorbic acid, colchicine, D-glucitol, BSO, bethanechol, and anandamide) formed one large cluster mainly affecting few readouts across the various endpoints. Each of these chemicals only caused effects

in 1-4 endpoints with little similarities among the chemicals. The only common endpoint shared among these chemicals is hyperactivity in the unstimulated behavior assay. Cytochalasin D, muscarine, and L-ascorbic acid caused hyperactivity at day 7 while BSO, bethanechol, and anandamide caused hyperactivity at day 12. It should be noted that this cluster contained two of our negative controls, D-glucitol and L-ascorbic acid. As expected, D-glucitol was inactive in all tested endpoints. L-ascorbic acid, however, was observed to have a concentration-independent effect on hyperactivity in the unstimulated behavior assay at day 7. Thus, these shared sporadic effects to induce hyperactivity in the unstimulated assay are difficult to interpret, as their toxicological significance is unclear. Interestingly, diazinon, the only OP in this cluster, only caused increased stickiness at both day 7 and 12 and did not show significant similarities to any of the other mechanistic controls. In the future, additional mechanistic control chemicals, and more concentrations of these, should be evaluated to clarify the significance of the observed phenotypes and delineate the mechanisms within this "other" cluster.

Developmentally selective OP toxicity characterized by comparison of toxicity in regenerating versus adult planarians

A unique strength of the planarian system is the ability to directly compare the toxicity seen in developing/regenerating planarians (Figure 6.2) with that in adult (intact) planarians (Figure 6.3) to identify effects which may be specific or more sensitive to development. When considering any endpoint, CPF, diazinon and malathion were found to be developmentally selective with the overall LOEL being lower in regenerating than in adult planarians (Table 6.3). When delving into the individual endpoints, this selectivity arises from increased sensitivity of regenerating planarians to body shape shapes (day 7/12) and/or increased stickiness (day 7/12) induced by these OPs. As mentioned above, these endpoints were also the most sensitive

endpoints for OP toxicity overall and thus appear to be sensitive indicators of OP DNT in planarians. In addition, although not determined to be selective on the chemical level, some endpoints affected by the OPs also showed developmental selectivity. For example, CPFO showed selectivity for day 7 body shape and increased stickiness, and DDVP showed selectivity for increased stickiness and unstimulated behavior, both at day 7. Parathion did not show any developmental selectivity and in fact even showed greater sensitivity in full rather than regenerating planarians resulting in overt systemic toxicity and lethality. In our previous work, we found that adult planarians generally tend to be more sensitive to lethality, and thus systemic toxicity (Hagstrom et al., 2015; Zhang et al., 2018), which we speculate may be the reason for the increased sensitivity of adult planarians to parathion.

These differences in toxic effects in adult and regenerating planarians were also apparent in the general toxicological profiles of the OPs. Unlike in regenerating planarians, effects of this chemical library in adult planarians were grouped into 3 broad categories: 1) cholinergic stimulation, 2) other, and 3) inactive (Figure 6.3). As in the regenerating tails, DDVP clustered with the cholinergic stimulators. However, all other OPs fell into the "other" cluster consisting of a mix of mechanistic controls, including the endocannabinoid stimulators, cytoskeletal disruptors, rotenone, and nicotine. Together, these data suggest that different mechanisms may be responsible for general versus developmental neurotoxicity induced by different OPs.

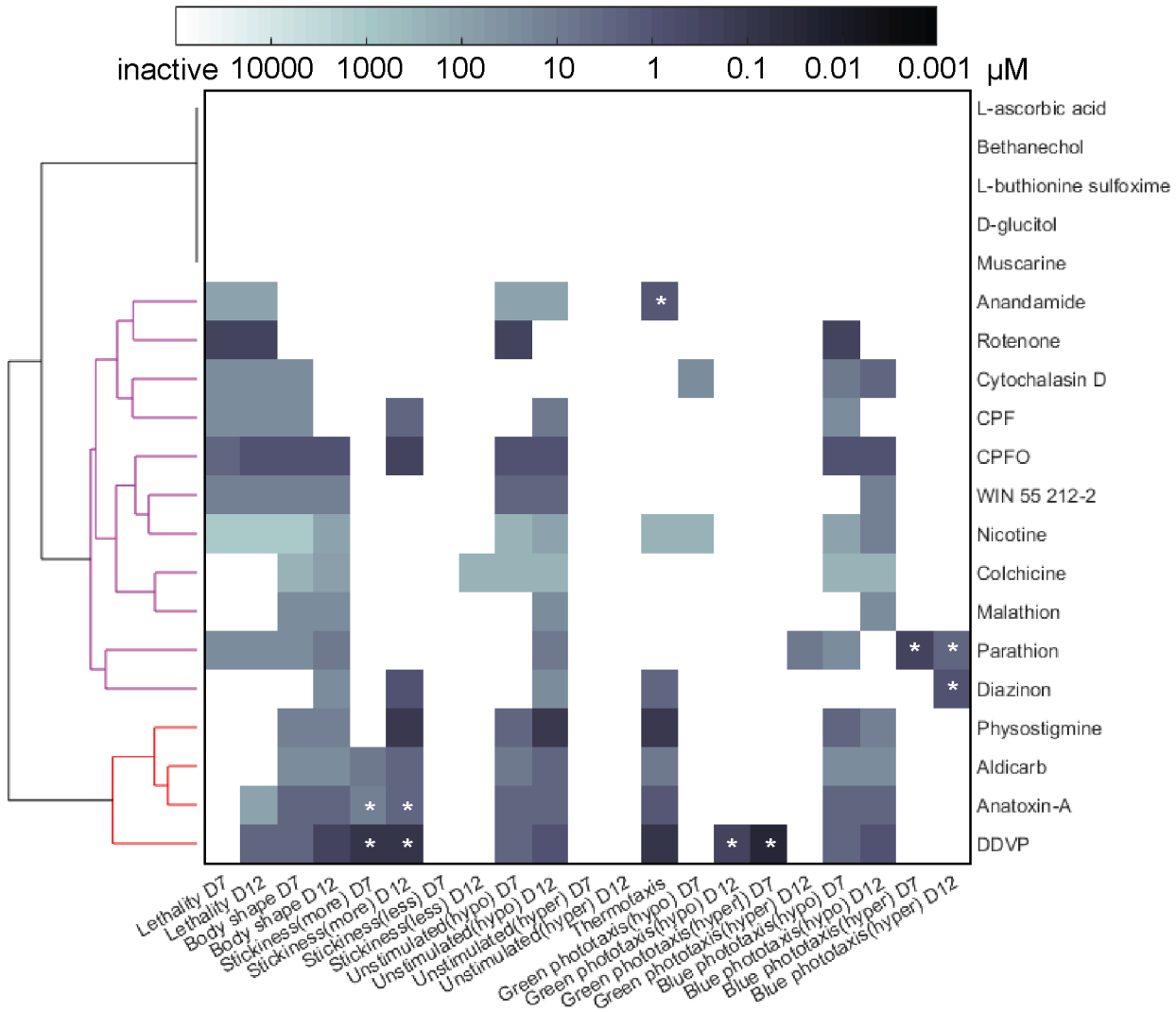

Figure 6.3. Full planarian toxicological profiles. Heat map of endpoints affected by each chemical in full planarians with LOEL color-coded. The chemicals' hits were clustered using Ward's method by calculating Euclidean distance between LOELs. LOELs defined by non-monotonic concentration responses are marked with *. Clusters were manually color-coded to aid comparison.

Table 6. 3. Developmental selectivity scores, quantified as the log($LOEL_{full}/LOEL_{regen}$), for endpoints shared in both worm-types. Positive scores (>0), shaded in grey, suggest the chemical was more potent in regenerating versus adult planarians for that endpoint. (-) indicates no activity was observed in that endpoint in regenerating planarians. If activity was observed in regenerating but not full planarians, the selectivity was estimated to be > than if the $LOEL_{full}$ was the highest tested concentration.

Chemical	Lethality D7	Lethality D12	Body shape D7	Body shape D12	↑ Stickiness D7	↑ Stickiness D12	↓ Stickiness D7	↓ Stickiness D12	↓ Unstimulated D7	↓ Unstimulated D12	↑ Unstimulated D7	↑ Unstimulated D12	Thermotaxis	↓ Green phototaxis D7	↓ Green phototaxisD12	↑ Green phototaxis D7	↑ Green phototaxis D12	↓ Blue phototaxis D7	↓ Blue phototaxis D12	↑ Blue phototaxis D7	↑ Blue phototaxisD12	overall
Chlorpyrifos	0	0	1.5	>0	>1	0	-	-	>0.5	-0.5	-	-	-	-	-	-	-	0	>0	-	-	0.5
Chlorpyrifos oxon	0	-0.5	0.5	-0.5	>0.5	-0.5	-	-	0	-	-	-	-	-	-	-	-	0	-	-	-	0
Diazinon	-	-	-	-	>2	0.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.5
Dichlorvos	-	-	0	-0.5	0.5	0	-	-	0.5	0	-	-	-1.0	-	-	-	-	0	-0.5	-	-	0
Malathion	-	-	1.0	0	>0.5	>0	-	-	>0.5	0.5	-	-	>0	-	-	-	-	-	-	-	-	1.0
Parathion	0	0	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	-	-	0	-1.0
Aldicarb	-	-	0	0	-	-0.5	-	-	-0.5	-0.5	-	-	-0.5	-	-	-	-	-0.5	-0.5	-	-	-0.5
Physostigmine	-	-	0	0.5	>1.5	-0.5	-	-	-0.5	-1.5	-	-	-2.0	-	-	-	-	-0.5	0	-	-	-0.5
Anatoxin-A	-	-	-0.5	0	-	0.5	>0.5	>0.5	-0.5	0	-	-	0	-	-	-	-	-0.5	0	-	-	0
Nicotine	0	0	0	-1.0	>2	-	-	-	-	0	-	-	0	0	-	-	-	-1.0	-1.5	-	-	0
Bethanechol	-	-	-	-	-	-	-	-	-	-	-	>0	-	-	-	-	-	-	-	-	-	>0
Muscarine	-	-	-	-	-	-	-	>1	-	-	>0.5	-	-	-	-	-	-	-	-	-	-	>1
Colchicine	-	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	-	-	-	-0.5
Cytochalasin D	-	0	-	-	>1.5	-	-	-	-	-	>2	>0.5	-	-	-	-	-	-	-	-	-	1.0
Anandamide	-	-	-	-	-	-	-	-	-	-	-	>0.5	-	-	>0.5	-	-	-	-	-	-	-1.5
WIN 55 212-2	0	0	0	0	-	-	-	>0	-	0	-	-	-	-	-	-	-	-	1.5	-	-	1.0
L-buthionine sulfoxime	-	-	-	-	-	-	-	-	-	-	-	>0	-	-	-	-	-	-	-	-	-	>0
Rotenone	-	-	>0.5	>0.5	-	>0.5	-	-	1.0	>0.5	-	>1.5	>0.5	-	-	-	-	-	>0	-	-	1.5
L-ascorbic acid	-	-	-	-	-	-	-	-	-	-	>0.5	-	-	-	-	-	-	-	-	-	-	>0.5
D-glucitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Summary

Our analysis showed that 5/6 OPs shared key phenotypic signatures, namely body shape changes and increased stickiness; however, the differential effects on other endpoints suggest compound-specific toxicities. DNT toxicological profiles were characterized by 1 of 3 mechanistic classes: 1) cholinergic overstimulation, exemplified by malathion and DDVP, 2) endocannabinoid and nicotinic AChR overstimulation, exemplified by CPF, CPFO and parathion, and 3) an unclassifiable "other" category containing diazinon. Despite being in a category by itself, diazinon's effects on increased stickiness, which was a common shared phenotype of almost all OPs and cholinergic stimulators, may suggest involvement of cholinergic toxicity. In fact, the most sensitive readouts to OP toxicity, body shape changes and increased stickiness, were also sensitive shared readouts of cholinergic toxicity induced by physostigmine, aldicarb, anatoxin-a and (to a lesser extent) nicotine, strongly suggesting these effects are cholinergic-dependent. To confirm this, the levels of AChE activity should be quantified in planarians exposed for 12 days to the LOEL of each OP to directly correlate these sensitive effects with AChE inhibition. When comparing effects on adult and regenerating planarians, body shape changes and increased stickiness were also the most developmentally selective readouts of OP toxicity suggesting specific DNT. Interestingly, although in mammals increased secretions due to cholinergic shock are a result of stimulation of muscarinic AChRs (Taylor, 2018), stimulation of muscarinic receptors by muscarine exposure in planarians resulted in decreased stickiness, suggesting less mucus secretion. Moreover, the shared effects of the nicotinic agonists on increased stickiness suggest mucus secretion may be regulated to a greater extent by nicotinic AChRs in planarians, but this finding requires further investigation. Furthermore, although the current analysis places the OPs into 1 of 3 mechanistic clusters, the

toxicological profiles of the different OPs likely results from concurrent effects on multiple targets. For example, CPF and CPFO, although clustering with the endocannabinoid/nicotine group, also have many similarities and shared readouts of the cholinergic cluster. Thus, toxicity induced by CPF/CPFO could be due to shared effects on both systems. Future analysis will focus on delineating these differences by creating a behavior map, linking endpoints and phenotypic signatures with mechanisms of action. These mechanistic connections could be further substantiated by screening additional mechanistic controls, as well as antagonists to the targets of interest, to better understand the functional significance of different perturbations of the target pathways. Moreover, co-exposure of OPs with well-characterized antagonists, such as the nicotinic AChR antagonist atropine, would allow us to confirm our proposed target-endpoint connections. These future steps would be performed with both regenerating and adult planarians; thus, allowing us to also clarify the observed differences between the toxicities of the two worm types. Lastly, previous studies suggest that due to the targeting of specific developmental events, such as synaptogenesis, certain developmental periods are more sensitive to OP exposure and that the timing of OP exposure affects which adverse outcomes are observed (Dam et al., 1999; Garcia et al., 2003; Qiao et al., 2002). Thus, future experiments comparing different exposure periods over the course of planarian regeneration could further dissect whether a critical vulnerable period exists for any of the planarian endpoints affected by OP exposure to connect effects on specific developmental milestones with their functional significance.

Together, these results demonstrate a strong link between effects on non-cholinergic targets and significant organismal adverse outcomes. Realization of the significance of compound-specific non-cholinergic OP toxicity is key to better understanding and protecting against environmental OP exposure, which are often administered in mixtures. Thus, this work

substantiates the need to evaluate the toxicity of different OPs alone and in mixtures to better understand any non-additive effects that may arise from effects and interactions on non-cholinergic targets.

Acknowledgements

A modified version of chapter 6 will be submitted for publication as a Research Article (Hagstrom, Danielle; Zhang, Siqi; and Collins, Eva-Maria S. “Comparative analysis of the mechanisms of organophosphorus pesticide developmental neurotoxicity in a freshwater planarian”). Siqi Zhang, Danielle Hagstrom and Eva-Maria S. Collins designed the experiments, interpreted the data and co-wrote the manuscript. Danielle Hagstrom set up all chemicals and experiments and analyzed the data. Siqi Zhang expanded the screening platform, performed the screening of the chemicals, and analyzed the data. Danielle Hagstrom and Siqi Zhang were the primary investigators and authors of this material.

References

Atwood, D. and Paisley-Jones, C. (2017). *Pesticides Industry Sales and Usage 2008 - 2012 Market Estimates*. Washington, DC.

Birkholz, T. R. and Beane, W. S. (2017). The planarian TRPA1 homolog mediates extraocular behavioral responses to near-ultraviolet light. *J. Exp. Biol.* **220**, 2616–2625.

Burke, R. D., Todd, S. W., Lumsden, E., Mullins, R. J., Mamczarz, J., Fawcett, W. P., Gullapalli, R. P., Randall, W. R., Pereira, E. F. R. and Albuquerque, E. X. (2017). Developmental neurotoxicity of the organophosphorus insecticide chlorpyrifos: from clinical findings to preclinical models and potential mechanisms. *J. Neurochem.* **142**, 162–177.

Buttarelli, F. R., Pontieri, F. E., Margotta, V. and Palladini, G. (2002). Cannabinoid-induced stimulation of motor activity in planaria through an opioid receptor-mediated mechanism. *Prog. Neuro-Psychopharmacology Biol. Psychiatry* **26**, 65–68.

Buttarelli, F. R., Pellicano, C. and Pontieri, F. E. (2008). Neuropharmacology and behavior in planarians: Translations to mammals. *Comp. Biochem. Physiol. - C Toxicol. Pharmacol.* **147**, 399–408.

Carr, R. L., Graves, C. A., Magnum, L. C., Nail, C. A. and Ross, M. K. (2014). Low level chlorpyrifos exposure increases anandamide accumulation in juvenile rat brain in the absence of brain cholinesterase inhibition. *Neurotoxicology* **43**, 82–89.

Casida, J. E. and Quistad, G. B. (2004). Organophosphate toxicology: safety aspects of nonacetylcholinesterase secondary targets. *Chem. Res. Toxicol.* **17**, 983–998.

Cebrià, F. (2007). Regenerating the central nervous system: how easy for planarians! *Dev. Genes Evol.* **217**, 733–48.

Crumpton, T. L., Seidler, F. J. and Slotkin, T. A. (2000). Is oxidative stress involved in the developmental neurotoxicity of chlorpyrifos? *Dev. Brain Res.* **121**, 189–195.

Dam, K., Garcia, S. J., Seidler, F. J. and Slotkin, T. A. (1999). Neonatal chlorpyrifos exposure alters synaptic development and neuronal activity in cholinergic and catecholaminergic pathways. *Brain Res. Dev. Brain Res.* **116**, 9–20.

Dam, K., Seidler, F. J. and Slotkin, T. A. (2000). Chlorpyrifos exposure during a critical neonatal period elicits gender-selective deficits in the development of coordination skills and locomotor activity. *Brain Res. Dev. Brain Res.* **121**, 179–87.

Dunkel, J., Talbot, J. and Schötz, E.-M. (2011). Memory and obesity affect the population dynamics of asexual freshwater planarians. *Phys. Biol.* **8**, 026003.

EUROSTAT (2016). *Agriculture, forestry and fishery statistics - 2016 edition*. (ed. Forti, R.) and Henrard, M.) Luxembourg, Belgium: European Union.

Flaskos, J. (2012). The developmental neurotoxicity of organophosphorus insecticides: a direct role for the oxon metabolites. *Toxicol. Lett.* **209**, 86–93.

Flaskos, J. (2014). The neuronal cytoskeleton as a potential target in the developmental neurotoxicity of organophosphorothionate insecticides. *Basic Clin. Pharmacol. Toxicol.* **115**, 201–8.

Gamaleddin, I. H., Trigo, J. M., Gueye, A. B., Zvonok, A., Makriyannis, A., Goldberg, S. R. and Le Foll, B. (2015). Role of the endogenous cannabinoid system in nicotine addiction: novel insights. *Front. psychiatry* **6**, 41.

Garcia, S. J., Seidler, F. J. and Slotkin, T. A. (2003). Developmental neurotoxicity elicited by prenatal or postnatal chlorpyrifos exposure: Effects on neurospecific proteins indicate changing vulnerabilities. *Environ. Health Perspect.* **111**, 297–303.

González-Alzaga, B., Lacasaña, M., Aguilar-Garduño, C., Rodríguez-Barranco, M., Ballester, F., Rebagliato, M. and Hernández, A. F. F. (2014). A systematic review of neurodevelopmental effects of prenatal and postnatal organophosphate pesticide exposure. *Toxicol. Lett.* **230**, 104–121.

Hagstrom, D., Cochet-Escartin, O., Zhang, S., Khuu, C. and Collins, E.-M. S. (2015). Freshwater planarians as an alternative animal model for neurotoxicology. *Toxicol. Sci.* **147**, 270–285.

Hagstrom, D., Cochet-Escartin, O. and Collins, E.-M. S. (2016). Planarian brain regeneration as a model system for developmental neurotoxicology. *Regeneration* **3**, 65–77.

Hagstrom, D., Hirokawa, H., Zhang, L., Radić, Z., Taylor, P. and Collins, E.-M. S. (2017). Planarian cholinesterase: in vitro characterization of an evolutionarily ancient enzyme to study organophosphorus pesticide toxicity and reactivation. *Arch. Toxicol.* **91**, 2837–2847.

Hagstrom, D., Zhang, S., Ho, A., Tsai, E. S., Radić, Z., Jahromi, A., Kaj, K. J., He, Y., Taylor, P. and Collins, E. M. S. (2018). Planarian cholinesterase: molecular and functional characterization of an evolutionarily ancient enzyme to study organophosphorus pesticide toxicity. *Arch. Toxicol.* **92**, 1161–1176.

Inoue, T., Yamashita, T. and Agata, K. (2014). Thermosensory signaling by TRPM is processed by brain serotonergic neurons to produce planarian thermotaxis. *J. Neurosci.* **34**, 15701–14.

Jiang, W., Duysen, E. G., Hansen, H., Shlyakhtenko, L., Schopfer, L. M. and Lockridge, O. (2010). Mice Treated with Chlorpyrifos or Chlorpyrifos Oxon Have Organophosphorylated Tubulin in the Brain and Disrupted Microtubule Structures, Suggesting a Role for Tubulin in Neurotoxicity Associated with Exposure to Organophosphorus Agents. *Toxicol. Sci.* **115**, 183–193.

Liu, J., Parsons, L. and Pope, C. (2013). Comparative effects of parathion and chlorpyrifos on

extracellular endocannabinoid levels in rat hippocampus: Influence on cholinergic toxicity. *Toxicol. Appl. Pharmacol.* **272**, 608–615.

Malinowski, P. T., Cochet-Escartin, O., Kaj, K. J., Ronan, E., Groisman, A., Diamond, P. H. and Collins, E.-M. S. (2017). Mechanics dictate where and how freshwater planarians fission. *Proc. Natl. Acad. Sci. U. S. A.* **114**, 10888–10893.

Mamczarz, J., Pescrille, J. D., Gavrushenko, L., Burke, R. D., Fawcett, W. P., DeTolla, L. J., Chen, H., Pereira, E. F. R., Albuquerque, E. X. and Albuquerque, E. X. (2016). Spatial learning impairment in prepubertal guinea pigs prenatally exposed to the organophosphorus pesticide chlorpyrifos: Toxicological implications. *Neurotoxicology* **56**, 17–28.

Moser, V. C. (1995). Comparisons of the acute effects of cholinesterase inhibitors using a neurobehavioral screening battery in rats. *Neurotoxicol. Teratol.* **17**, 617–625.

Muñoz-Quezada, M. T., Lucero, B. A., Barr, D. B., Steenland, K., Levy, K., Ryan, P. B., Iglesias, V., Alvarado, S., Concha, C., Rojas, E. and Vega, C. (2013). Neurodevelopmental effects in children associated with exposure to organophosphate pesticides: a systematic review. *Neurotoxicology* **39**, 158–168.

Nishimura, K., Kitamura, Y., Taniguchi, T. and Agata, K. (2010). Analysis of motor function modulated by cholinergic neurons in planarian Dugesia japonica. *Neuroscience* **168**, 18–30.

Paskin, T. R., Jellies, J., Bacher, J. and Beane, W. S. (2014). Planarian phototactic assay reveals differential behavioral responses based on wavelength. *PLoS One* **9**, e114708.

Pope, C. N. (1999). Organophosphorus pesticides: do they all have the same mechanism of toxicity? *J. Toxicol. Environ. Health. B. Crit. Rev.* **2**, 161–81.

Pope, C., Karanth, S. and Liu, J. (2005). Pharmacology and toxicology of cholinesterase inhibitors: uses and misuses of a common mechanism of action. *Environ. Toxicol. Pharmacol.* **19**, 433–446.

Qiao, D., Seidler, F. J., Padilla, S. and Slotkin, T. A. (2002). Developmental neurotoxicity of chlorpyrifos: what is the vulnerable period? *Environ. Health Perspect.* **110**, 1097–103.

Rauh, V., Arunajadai, S., Horton, M., Perera, F., Hoepner, L., Barr, D. B. and Whyatt, R. (2011). Seven-year neurodevelopmental scores and prenatal exposure to chlorpyrifos, a common agricultural pesticide. *Environ. Health Perspect.* **119**, 1196–1201.

Ribeiro, P., El-Shehabi, F. and Patocka, N. (2005). Classical transmitters and their receptors in flatworms. *Parasitology* **131**, S19-40.

Richendrfer, H. and Creton, R. (2015). Chlorpyrifos and malathion have opposite effects on behaviors and brain size that are not correlated to changes in AChE activity. *Neurotoxicology* **49**, 50–58.

Ross, K. G., Currie, K. W., Pearson, B. J. and Zayas, R. M. (2017). Nervous system development and regeneration in freshwater planarians. *Wiley Interdiscip. Rev. Dev. Biol.* **6**, 1–26.

Russom, C. L., LaLone, C. A., Villeneuve, D. L. and Ankley, G. T. (2014). Development of an adverse outcome pathway for acetylcholinesterase inhibition leading to acute mortality. *Environ. Toxicol. Chem.* **33**, 2157–2169.

Shelton, J. F., Geraghty, E. M., Tancredi, D. J., Delwiche, L. D., Schmidt, R. J., Ritz, B., Hansen, R. L. and Hertz-Picciotto, I. (2014). Neurodevelopmental disorders and prenatal residential proximity to agricultural pesticides: the CHARGE study. *Environ. Health Perspect.* **122**, 1103–9.

Singh, N., Lawana, V., Luo, J., Phong, P., Abdalla, A., Palanisamy, B., Rokad, D., Sarkar, S., Jin, H., Anantharam, V. and Kanthasamy, A.G. (2018). Organophosphate pesticide chlorpyrifos impairs STAT1 signaling to induce dopaminergic neurotoxicity: Implications for mitochondria mediated oxidative stress signaling events. *Neurobiol. Dis.* **117**, 82–113.

Slotkin, T. A., Levin, E. D. and Seidler, F. J. (2006). Comparative developmental neurotoxicity of organophosphate insecticides: Effects on brain development are separable from systemic toxicity. *Environ. Health Perspect.* **114**, 746–751.

Slotkin, T. A., Skavicus, S. and Seidler, F. J. (2017). Diazinon and parathion diverge in their effects on development of noradrenergic systems. *Brain Res. Bull.* **130**, 268–273.

Taylor, P. (2018). Anticholinesterase agents. In *Goodman and Gilman's The Pharmacological Basis of Therapeutics* (ed. Laurence L Brunton), pp. 163–176. San Francisco: McGraw Hill Education.

Yang, D., Howard, A., Bruun, D., Ajua-Alemanj, M., Pickart, C. and Lein, P. J. (2008). Chlorpyrifos and chlorpyrifos-oxon inhibit axonal growth by interfering with the morphogenic activity of acetylcholinesterase. *Toxicol. Appl. Pharmacol.* **228**, 32–41.

Yen, J., Donerly, S., Linney, E. A., Levin, E. D. and Linney, E. A. (2011). Differential acetylcholinesterase inhibition of chlorpyrifos, diazinon and parathion in larval zebrafish. *Neurotoxicol. Teratol.* **33**, 735–741.

Zarei, M. H., Soodi, M., Qasemian-Lemraski, M., Jafarzadeh, E. and Taha, M. F. (2015). Study of the chlorpyrifos neurotoxicity using neural differentiation of adipose tissue-derived stem cells. *Environ. Toxicol.*

Zhang, S., Hagstrom, D., Hayes, P., Graham, A. and Collins, E.-M. S. (2018). Multi-behavioral endpoint testing of an 87-chemical compound library in freshwater planarians. *Toxicol. Sci.*

Chapter 7. Conclusion and outlook

Conclusion

In this dissertation, I established and evaluated an automated high-throughput screening (HTS) whole-animal platform using the freshwater planarian *Dugesia japonica* to study developmental neurotoxicity.

In chapter 1, I addressed the urgent need of developing more alternative models, amenable to HTS, to accelerate the hazard assessment. Asexual freshwater planarians were introduced as a new alternative animal model to study developmental neurotoxicity. As planarians are small, inexpensive, easy to maintain and regenerate fast, they are amenable to HTS. More importantly, because of the similar size, adult and regenerating planarians can be compared in parallel with same assays, which is a unique strength of this system for developmental neurotoxicity testing. Various quantifiable behaviors provide the opportunity to study distinct neuronal functions.

In chapter 2, as a proof of concept, I introduced the semi-automated screening system we developed to assess the toxicity of 9 known neurotoxicants and a neutral agent, glucose, on both adult and regenerating planarians. By quantifying the effects on different endpoints, including viability, unstimulated behavior (gliding), stimulated behavior (thermotaxis), regeneration and brain structure, we found that different toxicants displayed varying toxicity with different levels of effects on various endpoints. Comparing the data of developing and adult animals, we also found that certain chemicals specially caused defects only in regenerating animals. Compared with more established alternative animal models, i.e. zebrafish and nematodes, the planarian system showed comparative sensitivity to the tested toxicants, suggesting that it is suitable as an alternative animal model to study developmental neurotoxicity. Additionally, this work

demonstrated the necessity of a fully automated screening system to improve the throughput and more quantitative endpoints to achieve specificity for developmental neurotoxicity.

Based on the concept and directions pointed out in chapter 2, in chapter 3, I introduced the fully automated planarian HTS platform that I built, which is capable of characterizing the effects of chemical compounds on various morphological and behavioral endpoints, including viability, eye regeneration, unstimulated behavior and stimulated behaviors (phototaxis, thermotaxis, scrunching) at two different time points (day 7 and 12). Using this HTS platform, we screened an 87-compound library of known and suspected neurotoxicants including pesticides, flame retardants, industrial compounds, drugs, polycyclic aromatic hydrocarbons (PAH) and presumptive negative controls, to evaluate the strengths and limitations of the planarian system. Through direct comparison of chemical bioactivity in regenerating and adult planarians, 13 chemicals including Bisphenol-A (BPA) and Chlorpyrifos (CPF) showed developmental selectivity in different endpoints. Furthermore, the large repertoire of behavioral readouts allows for the differentiation of defects between neurotoxicity and overt systemic toxicity. We detected sublethal behavioral effects in some chemicals, such as the pesticide aldicarb.

In chapter 4, I discussed our comparative analysis between the planarian and zebrafish systems using the same 87-compound library. We compared bioactive hits across endpoints, including developmental delay, morphological abnormality, viability and altered behavioral endpoints in both systems. We found that the planarian data were highly concordant with the developing zebrafish data and enriched the toxicological profiles by detecting additional unique behavioral endpoint hits. We showed that the two models are complementary to each other and

illustrated the value of dual alternative animal system testing to prioritize chemicals by providing a broader coverage of data readouts and validation of sensitivities.

In chapter 5, I discussed our work to further evaluate the robustness and reproducibility of the current planarian HTS system by screening a 15-flame retardant (FR)–specific library. We compared the results of this screen with the results of the same FR set in the previous screen of the 87-compound library to evaluate the robustness of the system. We showed that using triplicates (n = 24 in total), this system is sufficiently robust on reproducing bioactivity data of chemicals. We found that the overall chemical hit-call did not differ regardless off the number of replicates used (>=3 replicates). And the lowest observed effect level in different endpoints remained largely the same. Moreover, we compared our results in planarians with the published data in zebrafish, nematodes and *in vitro* cell-based models and found that planarians displayed high concordance and comparable sensitivity to other alternative models. Thus, we further validated the value of the planarian system for a toxicity testing battery approach with other diverse models. The comparative analysis also illustrated further the importance of using standardized methodology on evaluating and prioritizing chemical potency in the battery approach, and understanding the mechanisms, such as the uptake level of the animal models.

In chapter 6, I further expanded the planarian HTS platform and used it to investigate the possible mechanisms of developmental toxicity in organophosphorus pesticides (OP). The screened library consisted of 6 OPs, 12 compounds known to activate pathways suggested in the literatures to be OP targets, and 2 negative controls. The screening platform was improved by adding more endpoints, including body shape, phototaxis with green and blue light, and measurement of stickiness (adhesion to the substrate. By comparing the toxicological profiles of these chemicals, we discovered possible mechanisms of OP developmental neurotoxicity by

linking known modes of action to functional toxicological outcomes. In this work, we provided mechanistic insight into how different OPs can cause different effects on developing animals and demonstrated the strong link between relevant molecular targets and the significant adverse functional outcomes. To gain more insight into the mechanism, a library of OP-mixtures can be screened to understand how different OPs interact with each other. This is important, since humans are frequently exposed to multiple OPs simultaneously due to their environmental abundance and use in mixtures.

Outlook and future directions

While we established and evaluated planarians as an alternative animal model for developmental neurotoxicity screening, a lot of work needs to be done for the planarian system to be regarded as an established and reliable system in the modern toxicology testing framework. Here, I will discuss the future extension of our work, from the general aspects of screening platform optimization, future screens to be conducted with this system, and the understanding of pharmacokinetic/pharmacodynamics in alternative animal models.

Planarian HTS screening platform

Although we have used the current screening platform to successfully screen a large number of compounds, it is limited in throughput and efficiency. Since the experimental assays are performed sequentially, fully screening a single plate, with morphology assay, unstimulated behavioral assay, stickiness assay, phototaxis, thermotaxis and scrunching assay, requires approximately 23 minutes at day 12. Although the screening platform is fully automated, moderate human supervision is necessary to assure the quality of screening. If any malfunction happens during the screening (i.e. the Fresnel lens is not placed on the plate properly), it needs to be fixed as soon as possible. Therefore, if we consider the maximum amount of screening time is 14 hours per day, the throughput of the current single unit of screening platform is 18 chemicals/36 plates per day. The current system design limits the ability of the system to screen larger-scale chemical library (i.e. >1,000 chemicals). To greatly improve the throughput, the screening platform should be upgraded to a next generation to perform the assays in parallel stages. For example, by using conveyor belt or multiple robotic arms in the system to transport the plates, different assays can be performed at the same time to assess different plates, which will largely increase the efficiency.

Secondly, as I discussed in chapters 2, 3 and 6, the large repertoire of morphological and behavioral endpoints allows us to assess chemical effects on a wide spectrum of developmental and neuronal functions in planarians. The current expanded platform included viability, 2 morphological endpoints (body shape and eye regeneration), 5 behavioral endpoints (hyperactivity/hypo activity locomotion, increased/decreased stickiness, hyper activity/hypoactivity phototaxis, thermotaxis and scrunching), for two time points (day 7 and day 12). More endpoints can be added to the screening platform, by integrating additional quantifiable assays (chemotaxis, vibration sensation assay, thigmotaxis, etc.) and extracting more endpoints from the current assays. For example, freshwater planarians are able to sense and response to a chemical gradient, such as food (Inoue et al., 2015).

Thirdly, the majority of image analysis in the current platform is fully automated, but some still requires manual checks. For example, the current semi-automated analysis for body shape still relies on researchers to determine various body shapes. A fully automated shape-recognition analysis is particularly challenging in planarians, as they lack a fixed silhouette and the body is extremely soft and flexible. But it is possible to utilize the supervised learning algorithm to classify different body features with large training sets, as shown in nematode (Wählby et al., 2012) and zebrafish (Jeanray et al., 2015). Another challenge in the current analysis is to differentiate and track fissioned planarian head and tail pieces in the same plate well. New techniques must be adopted to largely improve the performance (efficiency and accuracy) of image processing and eventually fully automate all analysis processes.

Lastly, chemical and plate preparation must be automated. Currently, it requires large amounts of manual work to amputate planarians, add chemicals to exposure plates, and seal the plates with sealing film. For example, in the screen of 87-compound library, we needed to

manually prepare 522 plates with 25,056 planarians, where half of animals (12,528 animals) were manually amputated to induce head regeneration. Such amount of manual work limited the throughput of the whole system and also introduced potential issues of inaccuracy. Therefore, an automated liquid handling machine and worm amputation machine must be developed or purchased to achieve necessary HTS. Additionally, a complete database to integrate chemical and plate information and experiment data should also be developed to better organize and track all necessary information of chemical/plate for HTS data. Finally, food and water quality has to be standardized, as we found that varying food batch increased a measurable variability on planarians' sensitivity to chemicals.

Future screens to be conducted with planarian system

Utilizing the planarian HTS platform, larger and more diverse libraries should be screened. As an alternative animal model to bridge *in vitro* and mammalian models, the planarian system should be used to screen larger-scale chemical library with existing toxicity information well studied in *in vitro* models, and characterize the potential link between toxicity pathways to whole-animal adverse functional outcomes. For example, the phase I library of ToxCast program in EPA (www.epa.gov) consists of approximately 300 chemicals well studied in *in vitro* models with hundreds of assays and many tested chemicals also have access to traditional toxicity data and human clinical data. By screening this large-scale library and comparing the results across different models, one can greatly improve the predictive model of different toxicities in planarian with diverse phenotypic toxicological profiles. In addition, as we found in the work of chapter 3 and 4, planarian system displayed different sensitivities to different chemical classes, like other organism models. Thus, screening larger libraries of diverse chemical classes is also necessary to learn the ability of planarian screening platform to predict the toxicity of different chemical

classes. Furthermore, importantly, to integrate diverse models into a battery approach to predict human-relevant toxicities, it is necessary to screen the same library in multiple different models and perform comparative analysis of toxicology to better understand how different models complement each other and find the appropriate strategy to integrate toxicity data across models. And more diverse models need to be integrated to add values to the battery approach.

pharmacokinetic/pharmacodynamics in alternative animal models

Challenges were noted in the comparative analysis of planarian system with other alternative models to evaluate concordance in the work of chapter 3, 4 and 5. One major challenge is to understand the chemical uptake/absorption level and bioavailability in the animals. For example, as in zebrafish, the reported chemical concentration in planarian is nominal water concentration, which differs from the internal chemical concentration inside the animal. Additionally, planarian has a barrier of protective mucus, which might defense against chemical absorption through the epidermis. Therefore, any approach to measure or estimate the internal concentration will shed more light to the direct comparison of effect of doses across different models. More insight into the model systems, such as presence of relevant toxicological targets, xenobiotic metabolism and chemical absorption, is also required to effectively connect toxicity to the relevant exposure in mammals and humans.

Additionally, since one of the unique strengths in planarian system is that many behaviors relay on different neuronal subpopulations, more understandings of the mechanism of these behaviors helps to better connect the disruption in molecular or cellular pathways with functional adverse outcomes.

In summary, the ultimate goal to expand this work should be to achieve higher throughput and robustness, and together with other models in the batter approach to provide more accurate prediction of human-relevancy toxicity and accelerate the hazard assessment in the 21st century.

References

Inoue, T., Hoshino, H., Yamashita, T., Shimoyama, S. and Agata, K. (2015). Planarian shows decision-making behavior in response to multiple stimuli by integrative brain function. *Zool. Lett.* **1**, 7.

Jeanray, N., Marée, R., Pruvot, B., Stern, O., Geurts, P., Wehenkel, L. and Muller, M. (2015). Phenotype Classification of Zebrafish Embryos by Supervised Learning. *PLoS One* **10**, e0116989.

Wählby, C., Kamentsky, L., Liu, Z. H., Riklin-Raviv, T., Conery, A. L., O'Rourke, E. J., Sokolnicki, K. L., Visvikis, O., Ljosa, V., Irazoqui, J. E., Golland, P., Ruvkun, G., Ausubel, M. F., and Carpenter, E. A.. (2012). An image analysis toolbox for high-throughput C. elegans assays. *Nat. Methods* **9**, 714–716.

Appendix 1: Freshwater planarian high-throughput screening platform

This fully automated screening platform was designed for high-throughput screening of chemicals using freshwater planarians for the study of developmental neurotoxicology. The current expanded version (Figure 1) is capable of performing six different assays on adult and regenerating planarians in 48-well plates, including morphology/lethality assay, unstimulated behavioral assay, and 4 stimulated behavioral assays (stickiness assay, phototaxis (blue/green light), thermotaxis and scrunching assay). This platform consists of one commercial robotic plate handler (Hudson Robotics, Springfield Township, NJ), three custom-built imaging systems and four custom-built assay stations. One imaging system is specially used for imaging morphology/lethality assay, which scans the plate in x- and y- directions and allows for high-resolution imaging of each individual animal. The other two imaging systems are both equipped with one single camera and used to image the entire microplate in other assays. All components in the platform are controlled by the computer in MATLAB (Mathworks), Labview (National Instruments), and Softlinx Lab Automation software (Hudson Robotics).

Currently, it takes about 23 minutes to screen one plate with all 6 different assays performed in series. By screening plates at two time points (day 7 and day 12), this platform can generate data of 24 endpoints for regenerating planarians, and 23 endpoints for full planarians, including both hyper-/hypo- activities. This screening platform is versatile and customizable, allowing for the expansion of current assays and the addition of more different assays.

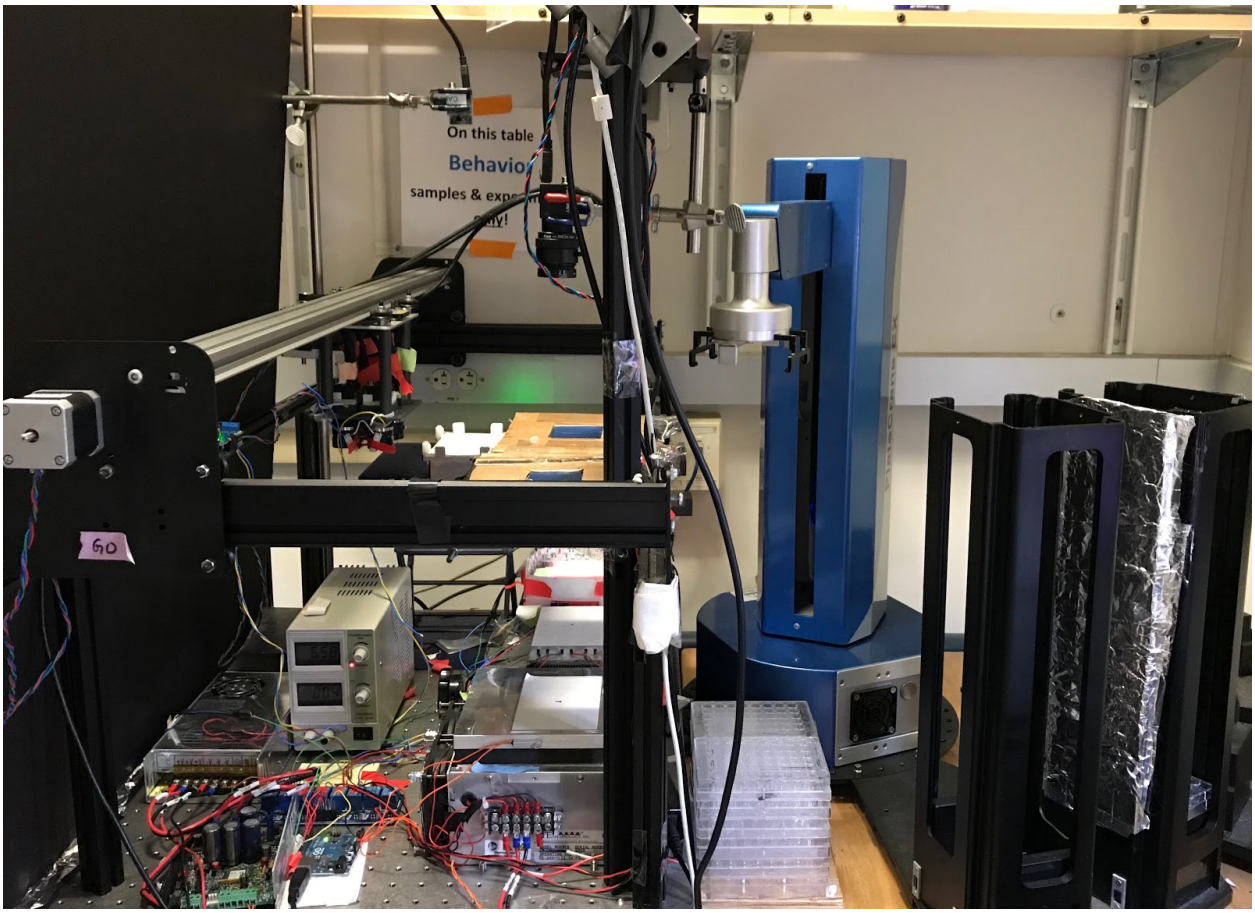

Figure 1. Expanded freshwater planarian high-throughput screening platform.

Appendix 2: Planarian cholinesterase: molecular and functional characterization of an evolutionarily ancient enzyme to study organophosphorus pesticide toxicity

This appendix 2 is a reformatted reprint of Hagstrom, Danielle; Zhang, Siqi; Ho, Alicia; Tsai, Eileen S.; Radić, Zoran; Jahromi, Aryo; Kaj, Kelson J.; He, Yingtian; Taylor, Palmer; and Collins, Eva-Maria S. "Planarian cholinesterase: molecular and functional characterization of an evolutionarily ancient enzyme to study organophosphorus pesticide toxicity", Archives of Toxicology, vol. 92, 2018.

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Abstract

The asexual freshwater planarian *Dugesia japonica* has emerged as a medium-throughput alternative animal model for neurotoxicology. We have previously shown that *D. japonica* are sensitive to organophosphorus pesticides (OPs) and characterized the in vitro inhibition pro le of planarian cholinesterase (DjChE) activity using irreversible and reversible inhibitors. We found that DjChE has intermediate features of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). Here, we identify two candidate genes (*Djche1* and *Djche2*) responsible for DjChE activity. Sequence alignment and structural homology modeling with representative vertebrate AChE and BChE sequences confirmed our structural predictions, and show that both DjChE enzymes have intermediate sized catalytic gorges and disrupted peripheral binding sites. *Djche1* and *Djche2* were both expressed in the planarian nervous system, as anticipated from previous activity staining, but with distinct expression pro les. To dissect how DjChE inhibition affects planarian behavior, we acutely inhibited DjChE activity by exposing animals to either an OP (diazinon) or carbamate (physostigmine) at 1 μM for 4 days. Both inhibitors delayed the reaction of planarians to heat stress. Simultaneous knockdown of both *Djche* genes by RNAi similarly resulted in a delayed heat stress response. Furthermore, chemical inhibition of DjChE activity increased the worms' ability to adhere to a substrate. However, increased substrate adhesion was not observed in *Djche1*/*Djche2* (*RNAi*) animals or in inhibitor-treated day 11 regenerates, suggesting this phenotype may be modulated by other mechanisms besides ChE inhibition. Together, our study characterizes DjChE expression and function, providing the basis for future studies in this system to dissect alternative mechanisms of OP toxicity.

Introduction

Organophosphorus pesticides (OP) are among the most agriculturally important and common pesticides used today. In the United States, 20 million pounds of OPs were used in 2012, accounting for 33% of all insecticides used (Atwood and Paisley-Jones 2017). Similarly, in 2014, Spain, France, Italy, Germany, and Poland, which together make up 72.7% of the European Union's pesticide sales (EUROSTAT 2016), had a combined usage of 4642 tons (~ 10 million pounds) of OPs according to the Food and Agriculture Organization of the United Nations (http://www.fao.org/faostat/en/#data/ RP). The primary shared mode of action of these pesticides is to inhibit the enzyme acetylcholinesterase (AChE), an essential regulator of cholinergic nerve transmission (Russom et al. 2014; King and Aaron 2015; Taylor 2017). Inhibition of AChE, which catalyzes the hydrolysis of the neurotransmitter acetylcholine (ACh), results in increased levels of synaptic ACh and subsequent overstimulation of nicotinic and muscarinic ACh receptors. Cholinergic toxicity is clinically manifested by decreased heart and respiration rates, increased secretions (sweating, lacrimation, and salivation), muscle tremors, and eventually paralysis and death (Eleršek and Filipic 2011; Russom et al. 2014; King and Aaron 2015; Taylor 2017). Because of its key role in regulating cognitive, peripheral autonomic, and somatic motor functions, AChE is also a common pharmacological target. For example, Alzheimer's disease, glaucoma, and myasthenia gravis have been treated with carbamate AChE inhibitors, such as physostigmine, and OPs, such as phospholine (echothiophate) iodide (Giacobini 2000; Pope et al. 2005; Taylor 2017).

At high concentrations, OPs are lethal to both insects and humans due to inhibition of AChE and subsequent cholinergic toxicity. However, there have been growing concerns that chronic, low concentration exposure to these pesticides can also cause harm. Epidemiological

studies have suggested a correlation between pesticide exposure and neurodegenerative diseases (Sánchez-Santed et al. 2016). Similar correlations have also been found linking prenatal and early life OP exposure to cognitive impairment in children (Muñoz-Quezada et al. 2013; Shelton et al. 2014; González-Alzaga et al. 2014).

In addition to inhibiting AChE function, studies have suggested that some chronic (Ray and Richards 2001; Terry 2012) and/or developmental (Timofeeva et al. 2008a, b) toxic outcomes may be independent of OPs' effects on AChE. This idea is corroborated by findings in in vivo and in vitro rat studies showing that OPs can have effects on a variety of cellular processes, such as cell signaling, oxidative stress, and axonal growth, at concentrations which do not significantly inhibit AChE (Slotkin and Seidler 2007; Yang et al. 2008). However, the degree that these secondary effects relate to specific toxic endpoints remains unclear.

ACh can also act as a neuromodulator to dynamically regulate the state of neurons, including but not limited to cholinergic neurons, in response to changing conditions (Picciotto et al. 2012). For example, feedback loops exist to regulate the levels of ACh synthesis, release, uptake, and receptor binding. Thus, chronic exposure to OPs may trigger compensatory mechanisms to adapt to chronically elevated ACh levels. The extent that adaptive mechanisms modulate specific toxic outcomes, and whether these mechanisms can be affected by secondary effects of OPs (Pope et al. 2005), warrant further investigation.

The freshwater planarian *Dugesia japonica* has recently emerged as a valuable in vivo model for neurotoxicity studies, with particular focus on neurodevelopment (Hagstrom et al. 2015, 2016). This asexual species naturally reproduces through transverse fission. Herein, the worm splits itself into two pieces which, due to a large population of adult stem cells (Rink 2013), subsequently regenerate all missing body structures, including the central nervous system

(CNS). In these animals, regeneration is the sole mechanism of neurodevelopment and shares fundamental processes with vertebrate neurodevelopment (Cebrià et al. 2002a, b; Cebrià and Newmark 2005; Umesono et al. 2011; Cowles et al. 2013). Distinct from other animal models, the similar sizes of full and regenerating planarians allows for a direct comparison of the effects of neurotoxicants on brain development and function with the same behavioral assays (Hagstrom et al. 2015, 2016). Using a custom planarian screening platform (Hagstrom et al. 2015), we showed that planarians are sensitive to OPs as subchronic exposure to sublethal concentrations of dichlorvos (10–500 nM) caused reduced rates of locomotion, with greater effects on regenerating rather than adult animals.

Furthermore, using activity measurements in planarian homogenates, we have recently demonstrated that planarian cholinesterase, DjChE, has intermediate characteristics of AChE and the closely related butyrylcholinesterase (BChE) (Hagstrom et al. 2017). Moreover, DjChE underwent similar rates of inhibition by OPs and carbamates as mammalian AChE, suggesting similar levels of sensitivity. We predicted that the enzyme(s) responsible for DjChE activity would be defined by a conserved catalytic triad and choline binding site, an active site gorge that is larger than that of AChE but smaller than BChE, and a disrupted peripheral anionic site. However, these predictions remained to be verified through structural analysis, and the in vivo expression pro le of the enzyme(s) was unknown. Moreover, a direct link between in vivo inhibition of DjChE activity and the functional con- sequences on planarian behavior is still missing. Herein, we verify our in vitro predictions by identifying and characterizing the expression and function of two candidate genes responsible for DjChE activity in vivo.

Using RNA interference (RNAi), we further compared the effects of simultaneous knockdown of both *Djche* genes with those induced by ChE inhibitors (diazinon and

physostigmine) on planarian locomotion, the animals' response to heat stress, and substrate adhesion. These endpoints were chosen based on our previous results that OPs can cause decreased planarian locomotion (Hagstrom et al. 2015), findings in nematodes that increased ACh levels caused heat stress tolerance (Furuhashi and Sakamoto 2016), and the use of hyper-secretions as one of the clinical hallmarks of cholinergic toxicity (Eleršek and Filipic 2011; Taylor 2017). Comparison of acute and subchronic developmental expo- sure of these endpoints suggests the existence of secondary effects on non-ChE targets to modulate the functional out-comes of OP toxicity.

Together, we structurally and functionally characterize DjChE and demonstrate a direct link between in vivo inhibition of DjChE activity and functional consequences on planarian behavior. This work therefore lays the foundation for the dissection of the mechanisms underlying OP toxicity in planarians.

Materials and Methods

Planarian culture

Freshwater asexual planarians of the species *Dugesia japonica* were used for all experiments. For behavioral experiments, animals used were 5.4 mm ± 1.1 mm (mean ± standard deviation) in length. Planarians were maintained in 1x Instant Ocean (IO, Blacksburg, VA) in Tupperware containers at 20°C in a Panasonic refrigerated incubator in the dark. Animals were fed organic chicken or beef liver 1-2x/week and cleaned twice a week when not used for experiments. Animals were starved for at least 5 days before experiments.

Identification and cloning of Djche

D. japonica homologs of acetylcholinesterase (AChE) were found using NCBI tBLASTn to query the deduced amino acid sequence of *Schistosoma mansoni* AChE (GenBank AAQ14321.1) (Bentley et al. 2003) against a *D. japonica* transcriptome. The transcriptome was assembled *de novo* from published sequencing data (Qin et al. 2011) using EBARDenovo (Chu et al. 2013). Two potential *ache* homologs were identified in *D. japonica* and crosschecked against the ESTHER protein database (Lenfant et al. 2013) to align most closely with acetylcholinesterase. Since we recently determined that *D. japonica* cholinesterase activity has characteristics of a hybrid AChE/BChE (Hagstrom et al. 2017), we termed these candidate sequences as *Djche1* and *Djche2*. The deduced amino acid sequences were determined from the longest open reading frame found using NCBI ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/). Sequence alignments were performed in JalView (Waterhouse et al. 2009).

RNA was extracted from recently amputated *D. japonica* head fragments using an RNeasy Mini Kit (Qiagen, Germantown, MD). Head cDNA was created using a SuperScript III

First Strand Synthesis Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA). Approximately 700 and 1000 bp fragments of *Djche1* and *Djche2*, respectively, were amplified from this cDNA by PCR using the following primers: Djche1_F: TCGAAACGCTATAATGGAATCCG, Djche1_R: AGGTTGGCAATGTTACTGTACG, Djche2_F: TTGGCAAGCTGATGGAAGTG, Djche2_R: CCAGCCGGTTATAGTTGAAGG. These fragments were subsequently cloned into the pPR-T4P vector.

Homology modeling of DjChE structure

Individual amino acid sequences of the two candidate DjChEs were submitted to Swiss-Model, a homology based 3D structure creation server (https://swissmodel.expasy.org/). The server searched its template library for evolutionary related structures matching the target sequence resulting in identification of several hundred potential templates. Template quality has been predicted from features of the target-template alignment and three of those with the highest quality were then selected for model building (Arnold et al. 2006; Benkert et al. 2011; Biasini et al. 2014). For both DjChE structures, *Torpedo californica* AChE was selected as the template (2cek and 2w6c, respectively). For comparisons in Figure 2, the 2w6c structure is shown. Additional details on model building can be found in Supplementary Materials.

In situ hybridization

Anti-sense digoxigenin (DIG) or fluorescein labeled probes were synthesized using T7 RNA polymerase essentially as described in (King and Newmark 2013). Planarian fixation and subsequent *in situ* hydridization were performed as in (King and Newmark 2013) with a few modifications: initial mucus removal was performed by treating with 2% hydrochloric acid in phosphate buffered saline (PBS) for 45 seconds with hand-inversion; animals were bleached overnight in 6% hydrogen peroxide in methanol under bright white light and subsequently

rehydrated in 50% MeOH/50% PBSTx (0.3% Triton-X 100 in PBS); and hybridization was performed at 60ºC overnight.

For co-localization experiments, a double fluorescent *in situ* hybridization (FISH) was performed using a combined POD-based tyramide development and AP-based Fast Blue development (Brown and Pearson 2015). Briefly, hybridization was performed concurrently with both DIG- and fluorescein-labeled riboprobes. Following post-hybridization washes, the samples were blocked in 5% horse serum and 0.5% Roche Western Blocking Reagent (RWBR, Roche, Indianapolis, IN) in MABT (150mM NaCl, 100mM Maleic Acid, 0.1% Tween 20, pH 7.5) at room temperature for 3-4 hours and treated overnight at 4˚C with a mix of anti-fluorescein-POD and anti-DIG-AP antibodies (both from Roche and diluted 1:2000 in 5% horse serum/0.5% RWBR). Following fluorescein tyramide development of the POD antibody, the samples were washed four times for 5-10 minutes with MABT. An AP-based Fast Blue development was then performed for colorimetric and fluorescent (far red) detection of the DIG-labeled riboprobe, as described in (Brown and Pearson 2015). Samples were mounted on glass slides and imaged on an inverted IX81 spinning disc confocal microscope (Olympus DSU) using an ORCA-ER camera (Hamamatsu Photonics) and Slidebook software (version 5, Intelligent Imaging Innovations, Inc).

Chemical Exposure

To analyze the effects of inhibition of ChE catalytic activity, planarians were exposed to 1μM physostigmine (eserine) or diazinon (both from Sigma-Aldrich, Saint Louis, MO). These concentrations were chosen because preliminary experiments determined they were not systemically toxic or lethal. Lack of systemic toxicity was demonstrated by the absence of lethality or morphological abnormalities (Fig. S1) for up to 12 days of exposure and by the

absence of regeneration delays (Fig. S4). Exposure solutions were prepared in IO water from 200X stocks solution in dimethyl sulfoxide (DMSO, Sigma-Aldrich) to have a final concentration of 0.5% DMSO. While others have suggested DMSO concentrations used with planarians should not exceed 0.1% (Pagán et al. 2006), we found 0.5% did not have a significant effect on planarian behavior (Hagstrom et al. 2015). Control animals were treated with 0.5% DMSO. Solutions were replaced daily to keep concentrations constant. During exposure, worms were kept in 12-well plates (Genesee, San Diego, CA) containing one worm and 1ml of chemical per well and stored in the dark at room temperature. Gliding and heat stress assays were performed on day 4 of exposure and stickiness assays on day 5. For experiments with regenerating animals, intact planarians were decapitated with an ethanol-sterilized razor blade. The tail pieces were placed in 12-well plates and exposed to inhibitor solutions within 1 hour of amputation. Gliding and heat stress assays were performed on day 11 of exposure/regeneration and stickiness assays on day 12. Experiments were performed in IO water.

Cholinesterase activity assays

Qualitative detection of ATCh or BTCh catalysis in fixed worms was performed as previously described (Zheng et al. 2011; Hagstrom et al. 2017), except staining incubation was decreased to 3.5 hours to gain the sensitivity needed to detect differences in activity in inhibitor-treated and knockdown animals.

To quantify the extent of ChE inhibition in inhibitor-treated planarians, 30 planarians were exposed to either 0.5% DMSO, 1μM diazinon, or 1μM physostigmine for 5 or 12 days, as described above. At the end of exposure, the planarians were washed three times with IO water and homogenized in 100μl 1% Triton X-100 (Sigma-Aldrich) in PBS as previously described (Hagstrom et al. 2017). Levels of acetylthiocholine (ATCh) catalysis (ChE activity) were

determined by an Ellman assay (Ellman et al. 1961) using 1mM ATCh (Sigma-Aldrich) as a substrate, as previously described (Hagstrom et al. 2017). Activity measurements were performed with at least 3 technical replicates per condition. Activity levels were normalized by protein concentration, determined by a Bradford Assay, and compared to the mean of the normalized levels in the DMSO controls in the same experiment (set as 100% activity). Data are shown as the mean and standard deviation of two independent experiments (biological replicates).

RNA interference (RNAi) experiments

Expression of *Djche1* and *Djche2* were knocked down individually and in combination by feeding planarians organic chicken liver mixed with *in vitro* transcribed dsRNA mixed with food coloring, per standard protocols (Rouhana et al. 2013). Negative control populations, denoted as *control (RNAi),* were fed organic chicken liver mixed with dsRNA of the *unc22* gene, a nonhomologous *C. elegans* gene. All RNAi treated populations were fed twice per week and cleaned three times per week. To speed up knock down, some RNAi worms were injected directly with the respective dsRNA (1µg/µl per gene). Injections were performed on intact animals daily for 4 consecutive days (Takano et al. 2007) using a Pneumatic PicoPump, Model PV 820 (World Precision Instruments, Sarasota, FL). One day after the last injection, the planarians were decapitated using an ethanol-sterilized razor blade. Animals were allowed to regenerate for 11 days before behavioral analysis.

Behavioral assays

Gliding

Six contact lens containers (Wöhlk Contactlinsen, Schönkirchen, Germany) containing one planarian each and 1.5 mL IO water were placed on a LED panel (Amazon, Seattle, WA).

The planarians were allowed to glide undisturbed for 10 minutes while imaging from above using a Basler camera (A601f-2, Basler, Germany), mounted on a ring stand. Assays were typically run with n=12 (2 sets of 6) animals per experiment for each condition. At least 2 independent experiments were run per condition. Gliding movies were analyzed as previously described in detail in (Hagstrom et al. 2015).

Heat stress

A single planarian was pipetted into 2 mL IO water into a 35 mm petri dish (CELLTREAT Scientific Products, Pepperell, MA). Of note, we also tried Falcon (Corning, NY) 35 mm petri dishes, but found that planarians in the CELLTREAT brand were easier to image because they spent relatively less time at the container edges. To create a high temperature environment, we used a peltier plate (TE Technology Inc., Traverse City, MI), which was controlled by a temperature controller (TE Technology, Inc.) and powered by an AC to DC power supply (Amazon). The plate was set to 52ºC and six dishes, with one planarian each, were heated for 10 minutes starting from room temperature. Thermistors were used to determine the dynamics of the aquatic temperature in the dishes over the course of the experiment (Fig. S1). The aquatic temperature stabilized after about 3 minutes to 30ºC and was consistent across all dishes and across multiple trials. The dishes were imaged from above using a Basler camera mounted to a ring stand. Lighting was provided via a red LED string light (Amazon) from above and surrounding the edges of the peltier. Assays were typically run with n=12 (2 sets of 6) animals per experiment and condition. At least 2 independent experiments were run per condition.

Analysis was performed using a custom MATLAB center-of-mass (COM) tracking script. The displacement of each worm across interlapping 12 second intervals was calculated in

MATLAB. Displacement was scaled by body length and displacements under 1 body length were empirically determined to correspond to movements which were primarily body shape changes. The proportion of displacements under 1 body length to all tracked displacements was determined and binned across one minute intervals. The median value for each condition is shown, with error bars representing the 25 and 75% quantiles.

Worm Substrate Adhesion ("Stickiness")

The stickiness of planarians was determined based on the worms' ability to adhere to a substrate as described in (Malinowski et al. 2017). In brief, an individual planarian was placed into a 3D printed plastic arena filled with 25ml of IO water and allowed to acclimate for approximately 2 mins. We then introduced a water flow and tested whether it was able to displace the worm from a fixed distance (~ 25mm). If displaced, the current flow rate was recorded with a Hall sensor (Amazon). If not displaced, the flow rate would be increased in discrete steps until displacement occurred.

Regeneration assay

The rate of blastema growth was determined as described in (Hagstrom et al. 2015). For chemical treatment, exposure began immediately (within 1 hour) after decapitation.

Statistical Analysis

Since all data for gliding, heat stress, and substrate adhesion experiments were not normally distributed, statistical analysis was done using the Wilcoxon rank sum test (Mann Whitney test) in MATLAB.

Results

D. japonica has two candidate genes encoding cholinesterase

We assembled a *D. japonica* transcriptome *de novo* using published sequencing data (Qin et al. 2011) as described in Materials and Methods. Two putative transcripts encoding cholinesterase were found using NCBI tBLASTn to query the deduced amino acid sequence of *Schistosoma mansoni* AChE (GenBank AAQ14321.1) (Bentley et al. 2003) against the *D. japonica* transcriptome. We named the two corresponding candidate genes *Djche1* and *Djche2.* The deduced amino acid sequences of these genes were aligned with representative amino acid sequences for vertebrate AChE and BChE from *Torpedo californica* (TcAChE) and human (HsBChE), respectively (Fig. 1).

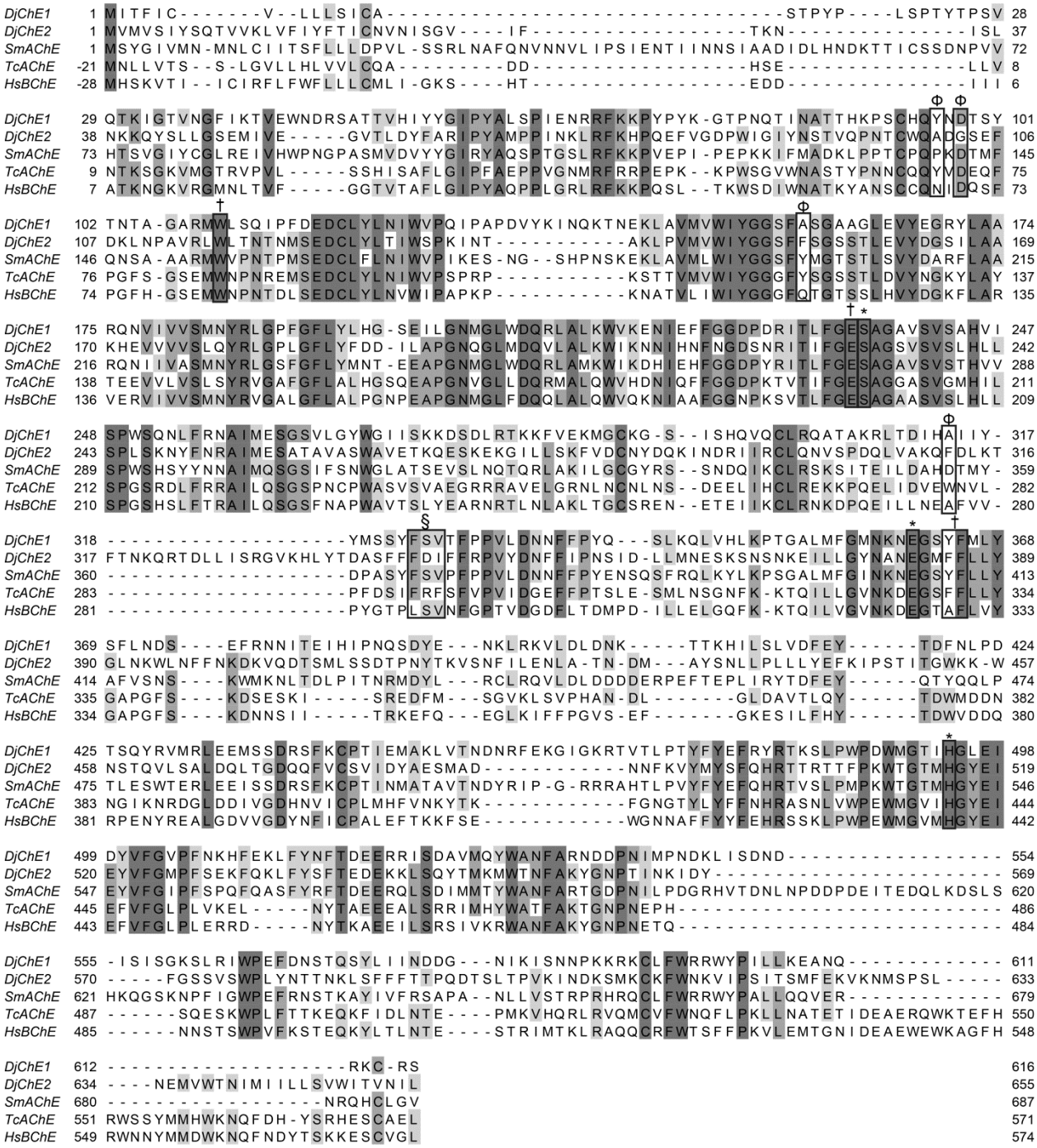

Figure 1. Candidate DjChEs show characteristics of both AChE and BChE. Alignment of deduced amino acid sequences of DjChE1 and DjChE2 with a representative vertebrate AChE (TcAChE), vertebrate BChE (HsBChE), and AChE from a related parasitic flatworm, *S. mansoni* (SmAChE). Note: for TcAChE and HsBChE, the leader signal peptide is shown but is not included in the numbering since it is not found in the mature polypeptides. Shading indicates level of conservation. Important structural residues are boxed and labeled: catalytic triad (*), acyl pocket (§), choline binding site (†), and peripheral anionic site (ϕ).

Both DjChE amino acid sequences contain essential catalytic residues for cholinesterase function, including the esterase-type catalytic triad (Ser200, Glu327, His440, numbering corresponding to TcAChE, per convention) and choline binding site (Trp84, Glu199, Phe330, Phe331). In agreement with our predictions based on *in vitro* inhibitor data (Hagstrom et al. 2017), both DjChE sequences seem to have intermediate characteristics between AChE and BChE (acyl pocket consisting of one phenylalanine, undefined peripheral anionic site).

We further evaluated the protein structure of the candidate planarian cholinesterases by performing homology modeling using the published structure of TcAChE (Paz et al. 2009) (Fig. 2). The homology-based structures of DjChE1 and DjChE2 similarly agree with our previous structural predictions (Hagstrom et al. 2017). Particularly, in both DjChE1 and DjChE2 structures, the catalytic triad and choline binding site are well conserved. Conversely, with only one (F288) of two commonly found phenylalanines and the substitution of the Arg289 "anchor" with a smaller side chain, the acyl pocket volume is much larger and more flexible than that of TcAChE. Lastly, several of the largely aromatic residues that define the vertebrate peripheral anionic site (Tyr70, Asp72, Tyr 121, Trp279) have been substituted with smaller aliphatic side chains in the planarian structures resulting in a wider gorge opening. In summary, both planarian cholinesterase candidate genes have hybrid features of both AChE and BChE, similar to other invertebrate cholinesterase (see Discussion) (Sanders et al. 1996; Bentley et al. 2005; Pezzementi et al. 2011).

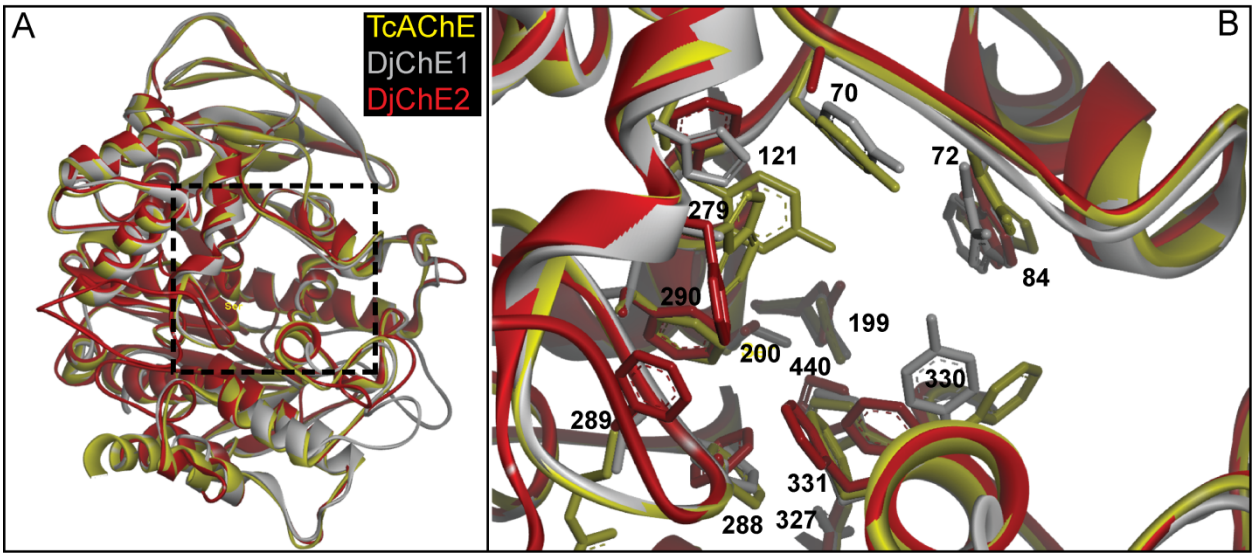

Figure 2. Homology modeling of planarian cholinesterase protein structure. **a** Whole protein structures of DjChE1 (grey) and DjChE2 (red) are overlaid with TcAChE (2w6c, yellow). Boxed area denotes the catalytic gorge. **b** Magnified view of boxed area in a. Important structural residues are labeled, with numbering based on TcAChE.

Djches are expressed in the planarian nervous system

Whole-mount fluorescent *in situ* hybridization (FISH) was performed to determine the expression patterns of *Djche1* and *Djche2* (Fig. 3). Similarly to the cholinergic marker, *Djchat* (Fig. 3A), *Djche1* is expressed widely throughout the planarian nervous system in both the anterior cephalic ganglion and ventral nerve cords (Fig. 3B). This mRNA expression profile agrees with cholinesterase activity stains which have shown cholinesterase enzymatic activity distributed throughout the planarian CNS (Hagstrom et al. 2017). *Djche2,* however, was found to be more ubiquitously expressed throughout the planarian body in a punctate pattern, with concentration of some puncta in the head region and along the nerve cords (Fig. 3C).

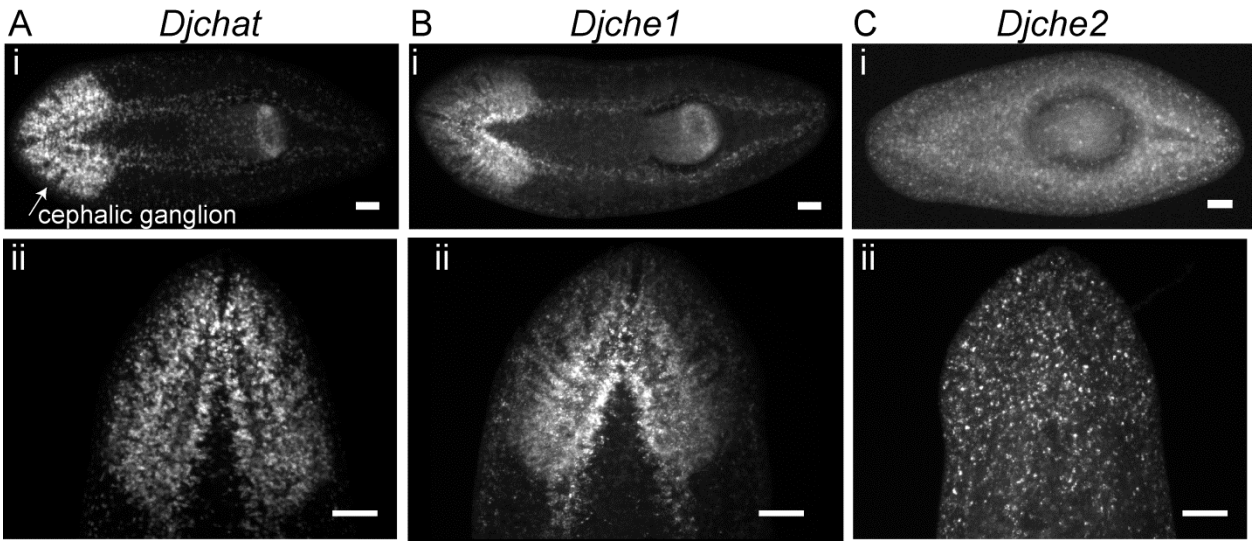

Figure 3. Planarian cholinesterases are expressed in the nervous system. Fluorescent *in situ* hybridization of *Djchat* (**a**), *Djche1* (**b**), and *Djche2* (**c**) showing the whole animal (i) or a maximum intensity projection of multiple planes in the head region (ii). Scale bars: 100μm.

Next, we performed multi-color FISH to determine the extent that these important regulators of the cholinergic system co-localize (Fig. 4). As expected from the single FISH, expression of *Djche1* extensively overlapped with expression of *Djchat* (Fig. 4A). *Djche2* also showed partial co-localization with both *Djchat* and *Djche1*, particularly in the medial arc of the cephalic ganglion (Fig. 4B-C).

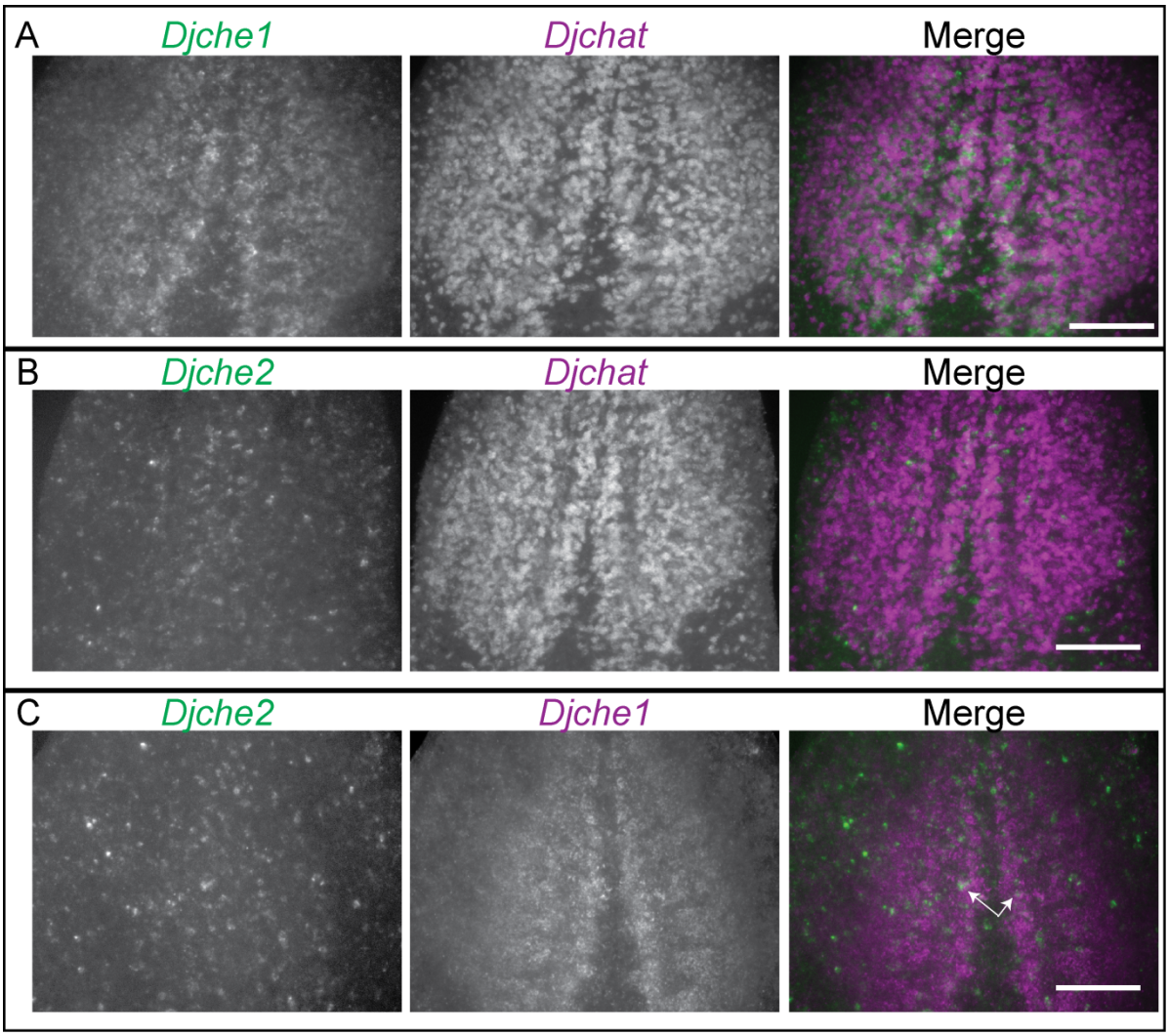

Figure 4. Planarian cholinesterases co-localize with each other and *Djchat* in the medial arc of the brain. Multicolor FISH for *Djche1* (green) and *Djchat* (magenta) (**a**), *Djche2* (green) and *Djchat* (magenta) (**b**), and *Djche2* (green) and *Djche1* (magenta) (**c**). Arrows denote co-localization in the medial arc domain. Scale bars: 100μm.

Inhibition of cholinesterase activity decreases sensitivity to heat stress

It has been previously shown in the nematode *Caenorhabditis elegans* that exogenous ACh exposure promotes thermotolerance. In these experiments, worms pre-cultured for 24 hours on plates containing ACh solution demonstrated increased survivability compared to controls after 10h incubation at 35°C (Furuhashi and Sakamoto 2016). Therefore, we assayed whether inhibition of ChE, which would increase synaptic ACh levels, affects planarians' response to heat stress. To this end, the animals' aquatic environment was slowly heated from room temperature to 30°C (Fig. S2) and the animals' reactions were monitored through video recordings (see Materials and Methods). Being higher than planarians' normal comfortable temperature range, 15-25 °C (Inoue et al. 2014), 30°C should induce heat stress while not induce scrunching, a planarian escape gait induced at 34-36°C (Cochet-Escartin et al. 2015). Solvent control animals (treated with 0.5% DMSO) reacted to the heat stress through frequent turns and head flailing, followed by decreased movement and eventual paralysis (Fig. 5A, Supplemental Video). This reaction was quantified by the fraction of time that the animals exhibited body shape changes rather than normal gliding behavior (see Materials and Methods). In control animals, the fraction of body shape changes gradually increased over time as the temperature rose and leveled out at approximately 0.9 once the temperature plateaued at 30° after 3 minutes (Fig. 5B).

To acutely inhibit DjChE activity, planarians were treated for 4 days with 1μM diazinon, an OP whose oxon metabolite efficiently inhibits DjChE activity *in vitro* (Hagstrom et al. 2017). Diazinon treated animals exhibited decreased sensitivity to heat stress, manifested in less body shape changes for a longer time (Supplemental Video). They eventually reached control levels by 10 minutes of heat exposure (Fig. 5A-B). To determine whether this phenotype was specific

to inhibition of ChE activity, we also exposed worms to physostigmine, a carbamate ChE inhibitor that has been previously shown to inhibit planarian ChE activity *in vitro* (Hagstrom et al. 2017). Moreover, acute exposure to at least 3μM physostigmine has been shown to cause planarians to contract (Nishimura et al. 2010). Similarly to diazinon, 4 day exposure to 1μM physostigmine caused a delayed reaction to heat stress (Fig. 5A-B). Activity stains confirmed that under these exposure conditions, diazinon and physostigmine significantly inhibited DjChE activity (Fig. 5C). Quantitative measurements of DjChE in homogenates of exposed planarians further confirmed significant inhibition of DjChE compared to solvent controls (Fig. S3).

To verify that differences in the heat stress response were not due to general motility differences, we assayed the unstimulated locomotion of these animals. At the used concentrations, physostigmine and diazinon caused a significant decrease in gliding speed (Fig. S4A). Notably, we previously observed a decrease in gliding speed of full planarians after exposure to dichlorvos for 8 days (Hagstrom et al. 2015), suggesting that this may be a shared phenotype of ChE inhibition.

Despite moving at a slower speed, the ChE inhibitor-treated animals had generally higher activity under heat stress than controls. Therefore, the heat stress phenotype is independent of the gliding phenotype.

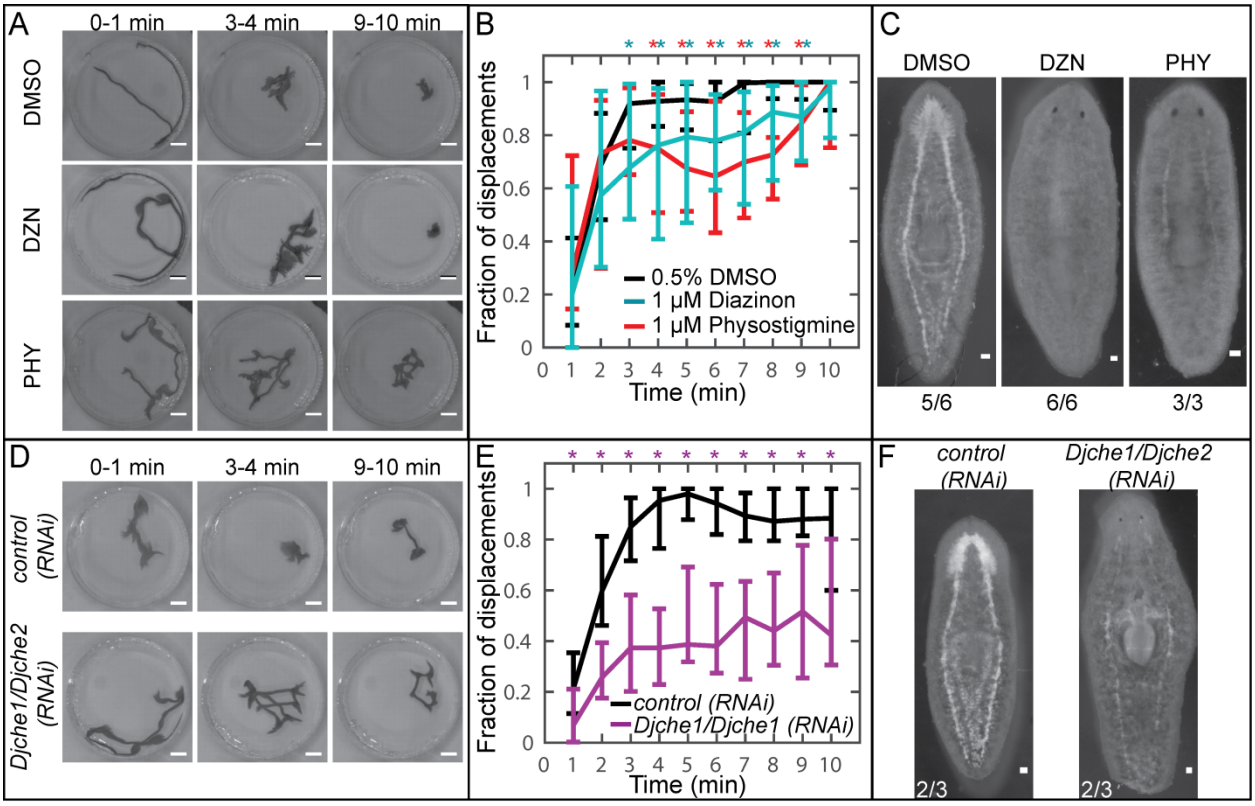

Figure 5. Inhibition of DjChE decreases sensitivity to heat stress **a** Representative minimum intensity projections over 1 minute intervals to show worm tracks of a 0.5% DMSO (DMSO) (top), 1µM diazinon (DZN, middle), and physostigmine (PHY, bottom) treated worm in response to heat stress. Note how during minutes 3-4, the DMSO-treated worm stays in one location with frequent turning (fan-like pattern in track) whereas the DZN and PHY-exposed planarians explore a larger area and have wider turns. **b** Diazinon and physotigmine treated animals undergo fewer and delayed body shape changes (as a fraction of all displacements tracked) than DMSO controls (n= 39, 46, 24 for DMSO, diazinon, and physostigmine, respectively). **c** ChE activity stains show inhibition of DjChE activity in 1µM diazinon and physostigmine treated animals. Numbers indicate how representative the staining is out of the number of animals assayed. **d** Representative minimum intensity projections over 1 minute intervals to show worm tracks of a *control (RNAi)* and *Djche1/Djche2 (RNAi)* animal in response to heat stress. **b** *Djche1/Djche2 (RNAi)* animals undergo fewer and delayed body shape changes (as a fraction of all displacements tracked) than *control (RNAi)* animals (n= 20 and 29 for *control (RNAi)* and *Djche1/Djche2 (RNAi)*, respectively). **c** ChE activity stains show loss of DjChE activity in *Djche1/Djche2 (RNAi)* animals. Numbers indicate how representative the staining is out of the number of animals assayed. Scale bars: 5mm (A, D), 100 µm (C, F). * indicates significant differences at the 5% level.

Knockdown of both Djches causes decreased sensitivity to heat stress

To determine whether the toxic outcomes of the ChE inhibitors were specific to their action on ChE, RNAi was used to simultaneously knockdown expression of both *Djche1* and *Djche2.* At first, RNAi was administered through feeding of dsRNA mixed with chicken liver. However, this technique remained inefficient at establishing consistent knockdown even after prolonged feedings (greater than 1 year). To increase the efficiency of knockdown, planarians which were previously fed RNAi liver were injected with dsRNA for both genes for 4 consecutive days. The animals were decapitated 1 day after the last injection and allowed to regenerate for 11 days before being assayed for behavioral phenotypes. This protocol was followed, because amputation and subsequent regeneration following dsRNA injection has been shown to increase knockdown efficiency in the newly regenerated tissue in planarians (Takano et al. 2007).

Djche1/Djche2 (RNAi) animals did not display any defects in regeneration when compared to *control (RNAi)* populations (Fig. S4). However, similarly to chemical inhibition of DjChE activity, *Djche1/Djche2 (RNAi)* animals were less sensitive to heat stress. They underwent dramatically less body shape changes as the temperature increased compared to *control (RNAi)* animals (Fig. 5D). Although the fraction of body shape changes did gradually increase over time, it never reached the same extent as in *control (RNAi)* animals (Fig. 5E). In contrast to acute chemical inhibition of DjChE, *Djche1/Djche2 (RNAi)* animals did not display noticeable differences in normal locomotion/gliding speed (Fig. S4). Knockdown of *Djche1* and *Djche2* mRNA were confirmed by whole-mount ISH (Fig. S5). We further confirmed that knockdown of the two putative *Djche* genes is sufficient to functionally knockdown DjChE

activity through staining of cholinesterase activity (Fig. 5F) and an Ellman assay of homogenized RNAi animals (Fig. S3).

Inhibition but not knockdown of Djche increases worm stickiness

When handling diazinon or physostigmine treated worms, we observed the animals tended to be "stickier" and often adhered to their substrate more strongly than control animals. Planarians secrete mucus for self-defense and locomotion, the latter of which is accomplished by cilia beating in a layer of secreted adhesive mucus (Martin 1978). Increased mucus secretion or changes in mucus composition in response to environmental stimuli can increase mucus production (Cochet-Escartin et al. 2015) and the worm's adhesion to its substrate ("stickiness") (Malinowski et al. 2017). To quantify the animals' stickiness, we dispelled a controlled stream of water at the animal and measured the flow rate necessary to dislodge the worm (Malinowski et al. 2017). In agreement with our qualitative assessment of increased stickiness, planarians which had been treated with 1μM diazinon or physostigmine for 5 days required larger flow rates to be dislodged, indicating increased stickiness and adhesion (Fig. 6A). Of note, although the distributions were significantly different from controls, the stickiness of inhibitor-treated planarians was much more variable than that of controls, possibly due to inter-worm variability in uptake or metabolism.

We next assayed whether *Djche1/Djche2 (RNAi)* animals also displayed increased stickiness to determine if this phenotype is specific to decreased DjChE activity. Unlike animals treated with the chemical inhibitors, *Djche1/Djche2 (RNAi)* animals did not demonstrate increased stickiness compared to *control (RNAi)* animals (Fig. 6B), suggesting that this effect may be modulated, in part or total, by mechanisms other than decreased DjChE activity.

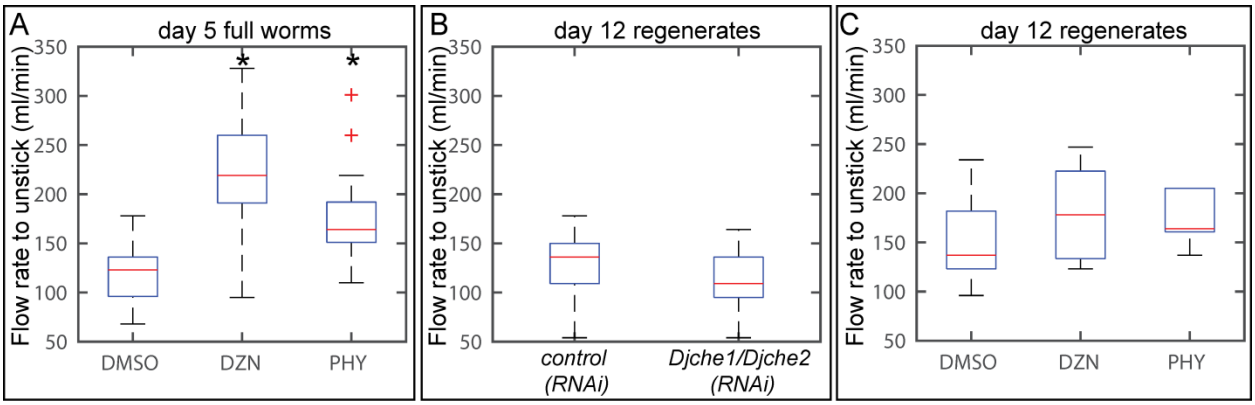

Figure 6. Diazinon and physostigmine, but not DjChE knockdown**,** increase worm adhesion ("stickiness"). Boxplot of the flow rate necessary to unstick worms from a substrate comparing worms exposed for 5 days to either **a** 0.5% DMSO (DMSO, n=46), 1µM diazinon (DZN, n=46), or 1µM physostigmine (PHY, n=23), **b** *control (RNAi)* (n=18) and *Djche1/Djche2 (RNAi)* (n=24) animals, or **c** regenerating tails exposed for 12 days to either 0.5% DMSO (DMSO, n=11), 1µM diazinon (DZN, n=9), or 1µM physostigmine (PHY, n=9). * indicates significant differences at the 5% level.

In summary, while acute chemical inhibition of DjChE activity causes effects on gliding speed, heat stress response, and substrate adhesion, knockdown of *Djche* gene expression only caused effects on the heat stress response. We therefore assayed whether absence of some behavioral effects in *Djche1/Djche2 (RNAi)* animals could be due to adaptation to decreased DjChE activity over time. To this end, we repeated our behavioral analysis on regenerating planarians exposed to either 1μM diazinon or physostigmine for 11-12 days. As with acute chemical inhibition and RNAi treatment, inhibitor-treated regenerating planarians exhibited a less pronounced heat stress response compared to control animals (Fig. S6) and had substantially less DjChE activity than control animals (Fig. S3). However, in contrast to acute inhibition, inhibitor-treated regenerating planarians were not significantly stickier than control animals (Fig. 6C). Particularly for diazinon-treated animals, the flow required to unstick the worms was significantly lower in regenerating animals compared to day 5 full animals. In addition, inhibitor-treated regenerating animals did not have reduced gliding speeds or any regeneration defects (Fig. S6). Thus, chemical inhibition of regenerating planarians recapitulated the effects seen with regenerating RNAi animals, but not those of acutely inhibited animals. Together, these data suggest that planarians may develop adaptive mechanisms to mitigate the effects of long-term cholinergic stimulation.

Discussion

Enzymatic properties of DjChE: sequence and structure

In this study, we have identified two potential gene sequences (*Djche1* and *Djche2*) responsible for cholinesterase activity in *D. japonica.* Our previous work characterizing the catalytic properties and inhibition profile of cholinesterase activity in planarian homogenates demonstrated that DjChE activity has hybrid properties of both AChE and BChE (Hagstrom et al. 2017). Both potential DjChE sequences identified in this study contain the features we previously predicted, namely: (a) classic esterase-type catalytic triad (Ser200, Glu 327 and His440), (b) an acyl pocket containing only one of two Phe (295 and 297), (c) a choline binding site containing Trp84, (d) disruption of a peripheral anionic site defined by Trp286, Tyr72, and Tyr124, and (e) fewer aromatic side chains lining the active center gorge compared to AChE (Figs. 1 and 2). Together these characteristics result in planarian cholinesterases with a larger acyl pocket and a wider gorge opening than vertebrate AChE. This is consistent with our previous observations that DjChE can catalyze the larger butyrylcholine substrate (although less efficiently than acetylcholine) and does not undergo substrate inhibition (Hagstrom et al. 2017). These qualities are common among cholinesterases from many invertebrates, including *Drosophila* (Gnagey et al. 1987)*, C. elegans* (Arpagaus et al. 1994)*, Schistosoma* (Bentley et al. 2003; Bentley et al. 2005), and some vertebrate species (Pezzementi et al. 2011) and may represent an ancestral cholinesterase before separation into the distinct AChE and BChE enzymes found in vertebrates (Pezzementi and Chatonnet 2010).

In planarian homogenates, we could not distinguish more than one distinct cholinesterase activity (Hagstrom et al. 2017). However, here we have identified two potential genes responsible for DjChE activity which are both actively expressed in *D. japonica*. Both genes

contain all the key enzymatic features described above, though sometimes achieved in different ways. Thus, they likely have similar catalytic properties and inhibitor affinities preventing us from distinguishing the two activities in crude homogenates. We cannot exclude the possibility, however, that one *Djche* may be much more highly expressed than the other and may account for the majority of the activity. Future experiments using recombinant enzymes would help answer whether there are any significant differences in the enzymatic properties of translated proteins expressed from these two genes.

Expression profiles of Djche

In vertebrates, AChE is encoded by a single gene but is alternatively spliced to produce different isoforms, differing only in their C-termini regions, each with distinct expression profiles and possibly different functions (Li et al. 1991; Taylor and Radić 1994; Soreq and Seidman 2001; Camp et al. 2010) Conversely, nematodes have three genes encoding AChE (*ace-1, -2, -3*) with distinct expression profiles and mostly non-redundant functions (Combes et al. 2003; Selkirk et al. 2005). Similarly to nematodes, *Djche1* and *Djche2* were found to have mostly non-overlapping expression profiles. D*jche1* is primarily expressed in the planarian nervous system with extensive co-localization with *Djchat*, suggesting this gene is expressed in cholinergic neurons. Conversely, *Djche2* is much more ubiquitously expressed throughout the planarian body with less spatial compartmentalization than *Djche1*. This spatial segregation could hint that the different *Djche* genes perform distinct functions, such as modulating ACh in the central versus the peripheral nervous systems, or discretely in synapses versus generalized release.

Interestingly, both *Djche* genes and *Djchat* were found to co-localize in neurons located in the medial arc of the planarian brain (Fig. 4). Several important regulators of planarian

neurogenesis and patterning are expressed in this region, including netrin (Cebrià et al. 2002b; Cebrià and Newmark 2005), hedgehog (Rink et al. 2009), and homeodomain transcription factors (Currie et al. 2016). In *S. mediterranea,* hedgehog and the homeodomain transcription factors *arx* and *nkx2.1* were all found to be expressed specifically in ventromedial cholinergic neurons. Knockdown of *arx* reduced the number of ventromedial cholinergic neurons specifically in adult animals, suggesting arx and the hedgehog machinery are necessary for maintenance of these neurons (Currie et al. 2016). Together, these data suggest that ventromedial neurons, such as those that co-express *Djchat* and the *Djche* genes, may be important for formation, patterning, and maintenance of the planarian brain. Studies in several animal models and cell culture systems have suggested that AChE may have morphogenic functions during neurodevelopment, which may or may not depend on catalysis of ACh (Bigbee et al. 2000; Biagioni et al. 2000; Paraoanu et al. 2006; Yang et al. 2008; Sperling et al. 2012; Layer et al. 2013). Consistent with the possibility that DjChE activity may regulate planarian brain formation, we previously found that subchronic exposure to high concentrations of the OP chlorpyrifos led to decreased brain size in regenerating but not full worms (Hagstrom et al. 2015). In this study, we did not observe any regeneration defects in either RNAi worms or chemically-treated worms. However, it is still possible that brain size defects were present, since this would have likely not been picked up by gross analysis of the blastema size. Thus, the role, if any, of DjChEs in planarian neurodevelopment remains to be discovered.

Functional consequences of decreased DjChE activity

Acute toxicity of OPs is primarily due to over-activation of the cholinergic system due to increased synaptic ACh levels and overstimulation of the nicotinic and muscarinic ACh receptors in the central and peripheral nervous systems (Pope et al. 2005; Russom et al. 2014; King and

Aaron 2015; Taylor 2017). However, it has long been recognized that these chemicals have other direct and indirect effects. For example, OPs have been shown to directly interact with other targets, including the ACh receptors, other esterases (e.g. neurotoxic esterase (NTE), carboxylesterase, etc.), and a host of other enzymes (e.g. lipases, proteases, acyl peptide hydrolase, etc.) (Clarke et al. 1994; Pope et al. 2005; Pancetti et al. 2007; Eleršek and Filipic 2011). These targets may also modulate the extent of cholinergic toxicity elicited by OP exposure by up- or down-regulating pre- and post-synaptic components involved in ACh synthesis, uptake, and binding (receptors) (Liu and Pope 1998; Pope et al. 2005). Importantly, actions on these secondary targets can vary dramatically between different OPs and can occur even at concentrations lower than necessary to inhibit AChE. Thus, it has been suggested that these secondary effects may play an important role in modulating the subacute and chronic effects of OPs, which can vary greatly depending on the inhibitor (Pope 1999; Casida and Quistad 2004; Pope et al. 2005; Eleršek and Filipic 2011). The manner and extent that these secondary effects play in the manifestation of specific toxic endpoints, however, is unclear.

We have previously reported that physostigmine and diazinon-oxon, the oxon metabolite of diazinon, are efficient inhibitors of DjChE activity *in vitro* (Hagstrom et al. 2017). However, *in vitro* inhibition is not necessarily predictive of *in vivo* inhibition capacity as other factors, such as the amount of inhibitor taken up by the animal, may modulate the actual concentration seen by the enzyme. In this study, we found that planarians, acutely exposed for 4-5 days to 1μM diazinon or physostigmine had substantial inhibition of DjChE activity, as seen qualitatively by activity staining (Fig. 5) and quantitatively by Ellman assays in homogenates of exposed worms (Fig. S3). Efficient *in vivo* inhibition by diazinon suggests that planarians are capable of bioactivation by cytochrome P450 of diazinon to diazinon-oxon, which is the active metabolite

responsible for AChE inhibition (Mutch and Williams 2006) Of note, in our hands, quantification of physostigmine-induced inhibition in homogenates underestimated the levels of inhibition when compared to the qualitative activity stains. This is likely due to the instability of the carbamylated enzyme as it can undergo rapid decarbamylation in the absence of physostigmine (Dawson 1994). For example, it has been reported that single dose exposure to physostigmine in Alzheimer's patients has a BChE inhibition half-life of 84 minutes (Asthana et al. 1995) . Thus, during the preparation of the homogenized sample, which takes approximately 1.5 hours, inhibited DjChE may be reactivated before activity measurements are made. However, in the activity stain in which animals are fixed immediately after exposure, inhibition by physostigmine can be accurately captured and demonstrated that substantial loss of DjChE activity had occurred (Fig. 5). Reactivation was likely a concern in both the day 5 full worms and day 12 regenerating worms, with differences between the two due to increased inhibition in the regenerates compared to the acute exposure (compare Fig. 5C and Fig S3D).

Despite significant loss of activity (greater than 95% in 1 μM diazinon treated animals), inhibitor-treated planarians were alive and generally healthy for up to 12 days of exposure with no overt morphological or regenerative defects (Fig. S1). Although AChE inhibition of 70-80% has been shown to be associated with lethality in birds, fish, and mammals (Russom et al. 2014), similar lack of systemic toxicity or lethality despite significant inhibition of AChE has been previously demonstrated. Exposure of zebrafish larvae for 5 days to varying concentrations of chlorpyrifos, diazinon, or parathion decreased AChE activity by more than 50-80% without inducing significant lethality (Yen et al. 2011). Moreover, in *C. elegans*, double mutants with nonfunctional *ace-1* and *ace-2*, which together account for approximately 95% of AChE activity,

are not lethal (Selkirk et al. 2005). Thus, in these species, as well as in planarians, it seems that very low levels of cholinesterase activity are sufficient to maintain viability.

In this study, both inhibitor-treated and *Djche1/Djche2 (RNAi)* animals displayed delayed and less reactive responses to heat stress, suggesting that increased thermotolerance is specific to loss of DjChE activity and subsequent overstimulation of the cholinergic system. This agrees with previous studies in *C. elegans*, which showed that excess ACh, either through exogenous ACh exposure or inhibition of AChE by neostigmine, led to increased thermotolerance, which was mediated by activation of a muscarinic receptor (Kalinnikova et al. 2013; Furuhashi and Sakamoto 2016).

Normal planarian locomotion is achieved through beating of cilia in a layer of secreted adhesive mucus (Martin 1978). Changes in mucus secretion or composition can change the adhesive properties of the worm, such as during physiological events such as fission (Malinowski et al. 2017) or in response to noxious stimuli to trigger an escape gait (Cochet-Escartin et al. 2015). Therefore, generally, an increase in worm stickiness would be considered an adverse effect on worm physiology and behavior. In this study, we found that while worms that were acutely treated with diazinon or physostigmine had increased stickiness, *Djche1/Djche2 (RNAi)* animals did not. This suggests that this endpoint may be modulated in part or total by some other mechanism besides decreased ChE activity. We have previously shown that the detergent Triton-X 100 increases mucus secretion and planarian stickiness (Malinowski et al. 2017) raising the possibility that increased mucus secretion and subsequent increased stickiness may be a nonspecific defense response to external toxicants. However, subchronic exposure (11-12 days) of regenerating planarians to the same concentrations of these ChE inhibitors did not elicit increased stickiness, compared to control animals (Fig. 6). Thus, as

this effect could be modulated, it is unlikely to be a general toxicant response. Additionally, inhibitor-treated regenerating planarians and RNAi animals did not show defects in gliding speed, although acute inhibitor-treated animals did. In our previous screen, we found that regenerating planarians were more sensitive than full worms to effects on gliding speed when treated with chlorpyrifos or dichlorvos (Hagstrom et al. 2015). Additionally, only dichlorvos, but not chlorpyrifos, caused a gliding speed defect in full animals (8 day exposure), suggesting that different effects beyond ChE inhibition may modulate how different OPs affect planarian locomotion. It is worth noting, however, that in the current study, we exchanged the chemical solutions daily to keep exposure conditions constant, while we did not exchange them in the previous screen. Together, these data suggest potential compensatory mechanisms may be activated in the regenerating animals to mitigate the long-term effects on stickiness and gliding speed.

Therefore, we propose that long-term overstimulation of the developing planarian cholinergic system may lead to adaptive mechanisms to gain tolerance to certain aspects of cholinergic toxicity, particularly increased stickiness and decreased gliding. In rats, down-regulation of the nicotinic and muscarinic ACh receptors has been proposed to be responsible for long-term tolerance to diazinon treatment (Ivanović et al. 2016) . Moreover, down-regulation of muscarinic receptors has been proposed to at least partially explain the surprisingly mild phenotypes of AChE knockout mice (Li et al. 2003). Increased secretions, including increased sweating, lacrimation, and salivation, due to overstimulation of muscarinic receptors are a hallmark of cholinergic toxicity (Pope et al. 2005; Eleršek and Filipic 2011; Taylor 2017). We have previously shown that increased planarian stickiness is correlated with an increase in mucus secretion (Malinowski et al. 2017). Therefore, we speculate that, while being induced through

ChE inhibition, increased worm stickiness may not be correlated directly with decreased ChE activity as compensatory mechanisms may allow planarians to adapt to long-term cholinergic overstimulation by down-regulating muscarinic receptors. The role of planarian muscarinic receptors in this process, whether modulation is due to direct or indirect effects of ChE inhibitors, and whether adaptation is specific to regenerating planarians remains to be verified.

Understanding of the potential role of non-ChE targets and effects in modulating ChE inhibitor toxicity is an important regulatory concern. Currently, levels of AChE inhibition are the gold standard biomarker to determine significant OP exposure (Kapka-Skrzypczak et al. 2011). However, growing evidence suggests that toxic outcomes may manifest from exposure to OP concentrations below those needed to inhibit AChE. This is of particular concern for chronic, low dose exposure and for prenatal exposure to the developing fetus (Pancetti et al. 2007). Here, we show that the wide repertoire of planarian morphological and behavioral endpoints, combined with accessible molecular biology techniques, enables us to dissect potential mechanisms underlying specific phenotypes of ChE inhibitor exposure. So far, however, we have only assayed a small subset of accessible behaviors based on endpoints that have previously been published to be affected by OP exposure in planarians or other systems. Thus, other effects of OP exposure may also exist that are not captured in this study. A comprehensive map quantifying the wide range of possible behaviors in planarians will be necessary for future studies aimed at elucidating the differential actions of OPs on neuronal function and behavior. As rates of OP inhibition of ChE are similar to mammals (Hagstrom et al. 2017) and the planarian brain contains many of the same important genes as the vertebrate brain, these mechanisms are likely to be conserved and could be further investigated in mammalian models. Together, these characteristics make planarians a well-suited model system to analyze OP toxicity.

Acknowledgements

The appendix 2, in full, is a reformatted reprint of the material as it appears in Hagstrom, Danielle; Zhang, Siqi; Ho, Alicia; Tsai, Eileen S.; Radić, Zoran; Jahromi, Aryo; Kaj, Kelson J.; He, Yingtian; Taylor, Palmer; and Collins, Eva-Maria S. "Planarian cholinesterase: molecular and functional characterization of an evolutionarily ancient enzyme to study organophosphorus pesticide toxicity", Archives of Toxicology, vol. 92, 2018. Permission to use this manuscript was granted to Siqi Zhang by Springer Nature. Danielle Hagstrom and Eva-Maria S. Collins designed the experiments and co-wrote the manuscript. Danielle Hagstrom, Siqi Zhang, Alicia Ho, Eileen S. Tsai, Aryo Jahromi, and Yingtian He performed the experiments and analyzed the associated data. Kelson Kaj assembled the *Dugesia japonica* transcriptome. Zoran Radić and Palmer Taylor performed analysis of the sequence and protein structure characteristics and contributed to writing and editing of the manuscript. Danielle Hagstrom was the primary investigator and author of this material.

References

Arnold K, Bordoli L, Kopp J, Schwede T (2006) The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics 22:195–201. doi: 10.1093/bioinformatics/bti770

Arpagaus M, Fedon Y, Cousin X, et al (1994) cDNA sequence, gene structure, and in vitro expression of ace-1, the gene encoding acetylcholinesterase of class A in the nematode Caenorhabditis elegans. J Biol Chem 269:9957–9965.

Asthana S, Greig NH, Hegedus L, et al (1995) Clinical pharmacokinetics of physostigmine in patients with Alzheimer's disease*. Clin Pharmacol Ther 58:299–309. doi: 10.1016/0009-9236(95)90246-5

Atwood D, Paisley-Jones C (2017) Pesticides Industry Sales and Usage 2008 - 2012 Market Estimates. Washington, DC

Benkert P, Biasini M, Schwede T (2011) Toward the estimation of the absolute quality of individual protein structure models. Bioinformatics 27:343–350. doi: 10.1093/bioinformatics/btq662

Bentley GN, Jones AK, Agnew A (2003) Mapping and sequencing of acetylcholinesterase genes from the platyhelminth blood fluke Schistosoma. Gene 314:103–112. doi: 10.1016/S0378-1119(03)00709-1

Bentley GN, Jones AK, Agnew A (2005) Expression and comparative functional characterisation of recombinant acetylcholinesterase from three species of Schistosoma. Mol Biochem Parasitol 141:119–123. doi: 10.1016/j.molbiopara.2005.01.019

Biagioni S, Tata AM, De Jaco A, Augusti-Tocco G (2000) Acetylcholine synthesis and neuron differentiation. Int J Dev Biol 44:689–97.

Biasini M, Bienert S, Waterhouse A, et al (2014) SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. Nucleic Acids Res 42:W252–W258. doi: 10.1093/nar/gku340

Bigbee JW, Sharma K V, Chan EL, Bögler O (2000) Evidence for the direct role of acetylcholinesterase in neurite outgrowth in primary dorsal root ganglion neurons. Brain Res 861:354–362. doi: 10.1016/S0006-8993(00)02046-1

Brown DDR, Pearson BJ (2015) One FISH, dFISH, three FISH: sensitive methods of whole-mount fluorescent in situ hybridization in freshwater planarians. In: Hauptmann G (ed) In Situ Hybridization Methods. Springer Science, New York, pp 127–150

Camp S, Zhang L, Krejci E, et al (2010) Contributions of selective knockout studies to understanding cholinesterase disposition and function. Chem Biol Interact 187:72–7. doi: 10.1016/j.cbi.2010.02.008

Casida JE, Quistad GB (2004) Organophosphate toxicology: safety aspects of nonacetylcholinesterase secondary targets. Chem Res Toxicol 17:983–998. doi: 10.1021/TX0499259

Cebrià F, Kudome T, Nakazawa M, et al (2002a) The expression of neural-specific genes reveals the structural and molecular complexity of the planarian central nervous system. Mech Dev 116:199–204. doi: 10.1016/S0925-4773(02)00134-X

Cebrià F, Nakazawa M, Mineta K, et al (2002b) Dissecting planarian central nervous system regeneration by the expression of neural-specific genes. Dev Growth Differ 44:135–146. doi: 10.1046/j.1440-169x.2002.00629.x

Cebrià F, Newmark PA (2005) Planarian homologs of netrin and netrin receptor are required for proper regeneration of the central nervous system and the maintenance of nervous system architecture. Development 132:3691–703. doi: 10.1242/dev.01941

Chu H-T, Hsiao WWL, Chen J-C, et al (2013) EBARDenovo: highly accurate de novo assembly of RNA-Seq with efficient chimera-detection. Bioinformatics 29:1004–1010. doi: 10.1093/bioinformatics/btt092

Clarke PBS, Reuben M, El-Bizri H (1994) Blockade of nicotinic responses by physostigmine, tacrine and other cholinesterase inhibitors in rat striatum. Br J Pharmacol 111:695–702. doi: 10.1111/j.1476-5381.1994.tb14793.x

Cochet-Escartin O, Mickolajczk KJ, Collins E-MS (2015) Scrunching: a novel escape gait in planarians. Phys Biol 12:55001. doi: 10.1088/1478-3975/12/5/056010

Combes D, Fedon Y, Toutant J-P, Arpagaus M (2003) Multiple ace genes encoding acetylcholinesterases of Caenorhabditis elegans have distinct tissue expression. Eur J Neurosci 18:497–512.

Cowles MW, Brown DDR, Nisperos S V, et al (2013) Genome-wide analysis of the bHLH gene family in planarians identifies factors required for adult neurogenesis and neuronal regeneration. Development 140:4691–702. doi: 10.1242/dev.098616

Currie KW, Molinaro AM, Pearson BJ (2016) Neuronal sources of hedgehog modulate neurogenesis in the adult planarian brain. Elife. doi: 10.7554/eLife.19735

Dawson RM (1994) Rate constants of carbamylation and decarbamylation of acetylcholinesterase for physostigmine and carbaryl in the presence of an oxime. Neurochem Int 24:173–182. doi: 10.1016/0197-0186(94)90104-X

Eleršek T, Filipic M (2011) Organophosphorus pesticides - mechanisms of their toxicity. In: Stoytcheva M (ed) Pesticides - The Impacts of Pesticides Exposure. Intech, pp 243–260

Ellman GL, Courtney KD, Andres V, Featherstone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7:88–95. doi: 10.1016/0006-2952(61)90145-9

EUROSTAT (2016) Agriculture, forestry and fishery statistics - 2016 edition. European Union, Luxembourg, Belgium

Furuhashi T, Sakamoto K (2016) Central nervous system promotes thermotolerance via FoxO/DAF-16 activation through octopamine and acetylcholine signaling in Caenorhabditis elegans. Biochem Biophys Res Commun 472:114–117. doi: 10.1016/j.bbrc.2016.02.076

Giacobini E (2000) Cholinesterase inhibitors: from the Calabar bean to Alzheimer therapy. In: Ezio Giacobini (ed) Cholinesterases and Cholinesterase Inhibitors. Martin Dunitz Ltd, London, pp 181–219

Gnagey AL, Forte M, Rosenberry TL (1987) Isolation and characterization of acetylcholinesterase from Drosophila. J Biol Chem 262:13290–13298.

González-Alzaga B, Lacasaña M, Aguilar-Garduño C, et al (2014) A systematic review of neurodevelopmental effects of prenatal and postnatal organophosphate pesticide exposure. Toxicol Lett 230:104–121. doi: 10.1016/j.toxlet.2013.11.019

Hagstrom D, Cochet-Escartin O, Collins E-MS (2016) Planarian brain regeneration as a model system for developmental neurotoxicology. Regeneration 3:65–77. doi: 10.1002/reg2.52

Hagstrom D, Cochet-Escartin O, Zhang S, et al (2015) Freshwater planarians as an alternative animal model for neurotoxicology. Toxicol Sci 147:270–285. doi: 10.1093/toxsci/kfv129

Hagstrom D, Hirokawa H, Zhang L, et al (2017) Planarian cholinesterase: in vitro characterization of an evolutionarily ancient enzyme to study organophosphorus pesticide toxicity and reactivation. Arch Toxicol 91:2837–2847. doi: 10.1007/s00204-016-1908-3

Inoue T, Yamashita T, Agata K (2014) Thermosensory signaling by TRPM is processed by brain serotonergic neurons to produce planarian thermotaxis. J Neurosci 34:15701–14. doi: 10.1523/JNEUROSCI.5379-13.2014

Ivanović SR, Dimitrijević B, Ćupić V, et al (2016) Downregulation of nicotinic and muscarinic receptor function in rats after subchronic exposure to diazinon. Toxicol Reports 3:523–530. doi: 10.1016/j.toxrep.2016.06.002

Kalinnikova TB, Shagidullin RR, Kolsanova RR, et al (2013) Acetylcholine deficiency in Caenorhabditis elegans induced by hyperthermia can be compensated by ACh-esterase inhibition or activation of GAR-3 mAChRs. Environ Nat Resour Res 3:98–113. doi: 10.5539/enrr.v3n3p98

Kapka-Skrzypczak L, Cyranka M, Skrzypczak M, Kruszewski M (2011) Biomonitoring and biomarkers of organophosphate pesticides exposure - state of the art. Ann Agric Environ Med 18:294–303.

King AM, Aaron CK (2015) Organophosphate and carbamate poisoning. Emerg. Med. Clin. North Am. 33:133–151.

King RS, Newmark PA (2013) In situ hybridization protocol for enhanced detection of gene expression in the planarian Schmidtea mediterranea. BMC Dev Biol. doi: 10.1186/1471-213X-13-8

Layer PG, Klaczinski J, Salfelder A, et al (2013) Cholinesterases in development: AChE as a firewall to inhibit cell proliferation and support differentiation. Chem Biol Interact 203:269–276. doi: 10.1016/j.cbi.2012.09.014

Lenfant N, Hotelier T, Velluet E, et al (2013) ESTHER, the database of the α/β-hydrolase fold superfamily of proteins: tools to explore diversity of functions. Nucleic Acids Res 41:D423-9. doi: 10.1093/nar/gks1154

Li B, Duysen EG, Volpicelli-Daley LA, et al (2003) Regulation of muscarinic acetylcholine receptor function in acetylcholinesterase knockout mice. Pharmacol Biochem Behav 74:977–986. doi: 10.1016/S0091-3057(03)00022-4

Li Y, Camp S, Rachinsky TL, et al (1991) Gene structure of mammalian acetylcholinesterase. Alternative exons dictate tissue-specific expression. J Biol Chem 266:23083–90.

Liu J, Pope CN (1998) Comparative presynaptic neurochemical canges in rat striatum following exposure to chlorpyrifos or parathion. J Toxicol Environ Heal Part A 53:531–544. doi: 10.1080/009841098159123

Malinowski PT, Cochet-Escartin O, Kaj KJ, et al (2017) Mechanics dictate where and how freshwater planarians fission.

Martin GG (1978) A new function of rhabdites: Mucus production for ciliary gliding. Zoomorphologie 91:235–248. doi: 10.1007/BF00999813

Muñoz-Quezada MT, Lucero BA, Barr DB, et al (2013) Neurodevelopmental effects in children associated with exposure to organophosphate pesticides: a systematic review. Neurotoxicology 39:158–168. doi: 10.1016/j.neuro.2013.09.003

Mutch E, Williams FM (2006) Diazinon, chlorpyrifos and parathion are metabolised by multiple cytochromes P450 in human liver. Toxicology 224:22–32. doi: 10.1016/J.TOX.2006.04.024

Nishimura K, Kitamura Y, Taniguchi T, Agata K (2010) Analysis of motor function modulated by cholinergic neurons in planarian Dugesia japonica. Neuroscience 168:18–30. doi: 10.1016/j.neuroscience.2010.03.038

Pagán OR, Rowlands AL, Urban KR (2006) Toxicity and behavioral effects of dimethylsulfoxide in planaria. Neurosci Lett 407:274–278.

Pancetti F, Olmos C, Dagnino-Subiabre A, et al (2007) Noncholinesterase effects induced by organophosphate pesticides and their relationship to cognitive processes: implication for the action of acylpeptide hydrolase. J Toxicol Environ Heal Part B Crit Rev 10:623–630. doi: 10.1080/10937400701436445

Paraoanu LE, Steinert G, Klaczinski J, et al (2006) On functions of cholinesterases during embryonic development. J Mol Neurosci 30:201–4. doi: 10.1385/JMN:30:1:201

Paz A, Xie Q, Greenblatt HM, et al (2009) The crystal structure of a complex of acetylcholinesterase with a bis-(−)- nor -meptazinol derivative reveals disruption of the catalytic triad. J Med Chem 52:2543–2549. doi: 10.1021/jm801657v

Pezzementi L, Chatonnet A (2010) Evolution of cholinesterases in the animal kingdom. Chem Biol Interact 187:27–33. doi: 10.1016/j.cbi.2010.03.043

Pezzementi L, Nachon F, Chatonnet A (2011) Evolution of acetylcholinesterase and butyrylcholinesterase in the vertebrates: An atypical butyrylcholinesterase from the medaka Oryzias latipes. PLoS One 6:e17396. doi: 10.1371/journal.pone.0017396

Picciotto MR, Higley MJ, Mineur YS (2012) Acetylcholine as a neuromodulator: cholinergic signaling shapes nervous system function and behavior. Neuron 76:116–129. doi: 10.1016/j.neuron.2012.08.036

Pope C, Karanth S, Liu J (2005) Pharmacology and toxicology of cholinesterase inhibitors: uses and misuses of a common mechanism of action. Environ Toxicol Pharmacol 19:433–46. doi: 10.1016/j.etap.2004.12.048

Pope CN (1999) Organophosphorus pesticides: do they all have the same mechanism of toxicity? J Toxicol Environ Heal Part B Crit Rev 2:161–181. doi: 10.1080/109374099281205

Qin YF, Fang HM, Tian QN, et al (2011) Transcriptome profiling and digital gene expression by deep-sequencing in normal/regenerative tissues of planarian Dugesia japonica. Genomics 97:364–371. doi: 10.1016/j.ygeno.2011.02.002

Ray DE, Richards PG (2001) The potential for toxic effects of chronic, low-dose exposure to organophosphates. Toxicol Lett 120:343–351. doi: 10.1016/S0378-4274(01)00266-1

Rink JC (2013) Stem cell systems and regeneration in planaria. Dev Genes Evol 223:67–84. doi: 10.1007/s00427-012-0426-4

Rink JC, Gurley KA, Elliott SA, Sánchez Alvarado A (2009) Planarian Hh Signaling Regulates Regeneration Polarity and Links Hh Pathway Evolution to Cilia.

Rouhana L, Weiss J a., Forsthoefel DJ, et al (2013) RNA interference by feeding in vitro-synthesized double-stranded RNA to planarians: Methodology and dynamics. Dev Dyn 242:718–730. doi: 10.1002/dvdy.23950

Russom CL, LaLone CA, Villeneuve DL, Ankley GT (2014) Development of an adverse outcome pathway for acetylcholinesterase inhibition leading to acute mortality. Environ Toxicol Chem 33:2157–2169. doi: 10.1002/etc.2662

Sánchez-Santed F, Colomina MT, Herrero Hernández E (2016) Organophosphate pesticide exposure and neurodegeneration. Cortex 74:417–426. doi: 10.1016/j.cortex.2015.10.003

Sanders M, Mathews B, Sutherland D, et al (1996) Biochemical and molecular characterization of acetylcholinesterase from the hagfish Myxine glutinosa. Comp Biochem Physiol , Part B Biochem Mol Biol 115:97–109. doi: 10.1016/0305-0491(96)00088-0

Selkirk ME, Lazari O, Hussein AS, Matthews JB (2005) Nematode acetylcholinesterases are encoded by multiple genes and perform non-overlapping functions. Chem Biol Interact 157–158:263–268. doi: 10.1016/j.cbi.2005.10.039

Shelton JF, Geraghty EM, Tancredi DJ, et al (2014) Neurodevelopmental disorders and prenatal residential proximity to agricultural pesticides: the CHARGE study. Environ Health Perspect 122:1103–9. doi: 10.1289/ehp.1307044

Slotkin TA, Seidler FJ (2007) Comparative developmental neurotoxicity of organophosphates in vivo: transcriptional responses of pathways for brain cell development, cell signaling, cytotoxicity and neurotransmitter systems. Brain Res Bull 72:232–274. doi: 10.1016/j.brainresbull.2007.01.005

Soreq H, Seidman S (2001) Acetylcholinesterase--new roles for an old actor. Nat Rev Neurosci 2:294–302. doi: 10.1038/35067589

Sperling LE, Klaczinski J, Schütz C, et al (2012) Mouse acetylcholinesterase enhances neurite outgrowth of rat R28 cells through interaction with laminin-1. PLoS One 7:e36683. doi: 10.1371/journal.pone.0036683

Takano T, Pulvers JN, Inoue T, et al (2007) Regeneration-dependent conditional gene knockdown (Readyknock) in planarian: Demonstration of requirement for Djsnap-25 expression in the brain for negative phototactic behavior. Dev Growth Differ 49:383–394. doi: 10.1111/j.1440-169X.2007.00936.x

Taylor P (2017) Anticholinesterase agents. In: Laurence L Brunton (ed) Goodman and Gilman's The Pharmacological Basis of Therapeutics, 13th edn. McGraw Hill, pp 239–254

Taylor P, Radić Z (1994) The cholinesterases: from genes to proteins. Annu Rev Pharmacol Toxicol 34:281–320. doi: 10.1146/annurev.pa.34.040194.001433

Terry AVJ (2012) Functional consequences of repeated organophosphate exposure: potential non-cholinergic mechanisms. Pharmacol Ther 134:355–65. doi: 10.1016/j.pharmthera.2012.03.001

Timofeeva OA, Roegge CS, Seidler FJ, et al (2008a) Persistent cognitive alterations in rats after early postnatal exposure to low doses of the organophosphate pesticide, diazinon. Neurotoxicol Teratol 30:38–45. doi: 10.1016/j.ntt.2007.10.002

Timofeeva OA, Sanders D, Seemann K, et al (2008b) Persistent behavioral alterations in rats neonatally exposed to low doses of the organophosphate pesticide, parathion. Brain Res Bull 77:404–411. doi: 10.1016/j.brainresbull.2008.08.019

Umesono Y, Tasaki J, Nishimura K, et al (2011) Regeneration in an evolutionarily primitive

brain--the planarian Dugesia japonica model. Eur J Neurosci 34:863–9. doi: 10.1111/j.1460-9568.2011.07819.x

Waterhouse AM, Procter JB, Martin DMA, et al (2009) Jalview Version 2--a multiple sequence alignment editor and analysis workbench. Bioinformatics 25:1189–1191. doi: 10.1093/bioinformatics/btp033

Yang D, Howard A, Bruun D, et al (2008) Chlorpyrifos and chlorpyrifos-oxon inhibit axonal growth by interfering with the morphogenic activity of acetylcholinesterase. Toxicol Appl Pharmacol 228:32–41. doi: 10.1016/j.taap.2007.11.005

Yen J, Donerly S, Linney EA, et al (2011) Differential acetylcholinesterase inhibition of chlorpyrifos, diazinon and parathion in larval zebrafish. Neurotoxicol Teratol 33:735–741. doi: 10.1016/j.ntt.2011.10.004

Zheng D-M, Xie H-Q, Wang A-T, Wu C-C (2011) The nerve system identificiation by histochemical localization of acetylcholinesterase in planarian Dugesia japonica. Chinese J Zool 45:68–75.