Rijksinstituut voor Volksgezondheid
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An inter-laboratory case study to determine the added value of the zebrafish light-dark transition test to predict developmental neurotoxicity: Report from OECD DNT Expert Group

Ellen Hessel

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Why Study Developmental Neurotoxicity?

- Prevalence of developmental neuronal disorders is increasing
- Role of chemical exposure is almost unknown
- The developing central nervous system (CNS) has a different susceptibility to chemical-induced injury than the adult CNS.
- CNS development is a complex process involving many different events within strictly controlled time frames and therefore each event might create a different window of vulnerability to chemical exposure.

Regulatory need for DNT

Current test guidelines: OECD-426 and 443

- Very expensive and time consuming
- Human relevance unknown
- Only 150 compounds tested

Need for integrated testing battery for DNT

- Screening more chemicals
- As a first screening step for prioritisation
- Human relevance is essential
- Based on mechanistic knowledge and AOPs

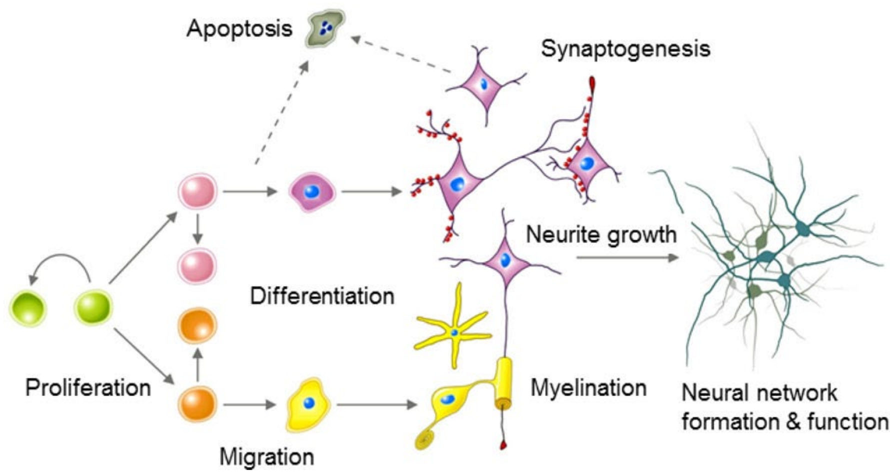
OECD develops a guidance document for DNT

Background OECD guidance document (GD) - IATA

- Aim: description of an *in vitro test battery* for developmental neurotoxicity based on key processes.
  - *How data could be interpreted and used to assess DNT.*

- Outline an integrated approach to testing and assessment (IATA) for the purposes for screening and prioritization or for hazard assessment.

- The Zebrafish behavioral assay could be one of the *tests.*



Aschner et al., 2017

Added value zebrafish

- Small fish used >4 decades in pharmaceutical research
  - Easy husbandry + strong historical database

- High human relevance
  - ~70% gene concordance; 80% concordance with drug targets
- Higher throughput
  - Many embryos + shorter life stages
- Embryos are transparent
  - Ease of internal observations

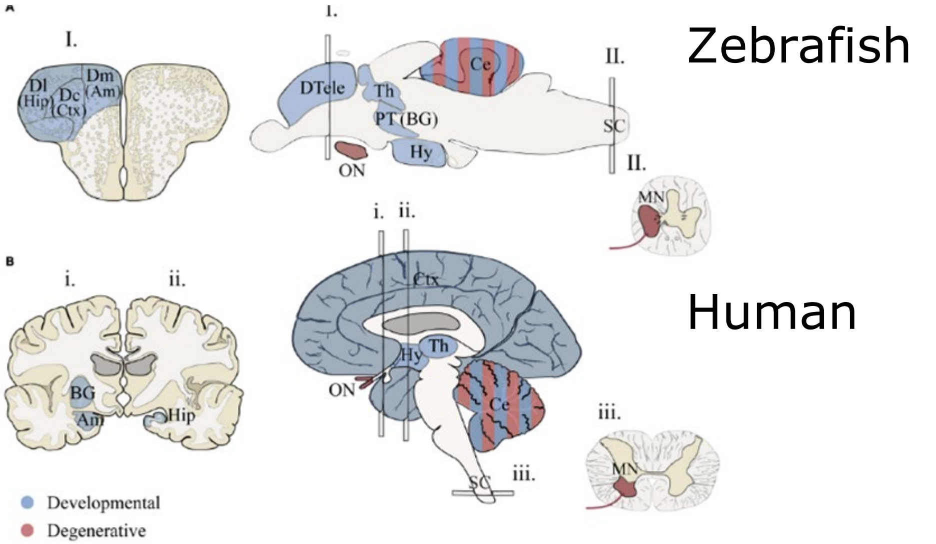
6 hpf **24 hpf** **48 hpf**

- Greater statistical power

  Large replicates (12 embryos) e.g. 3 reps = 3x12 = 36 embryos/dose

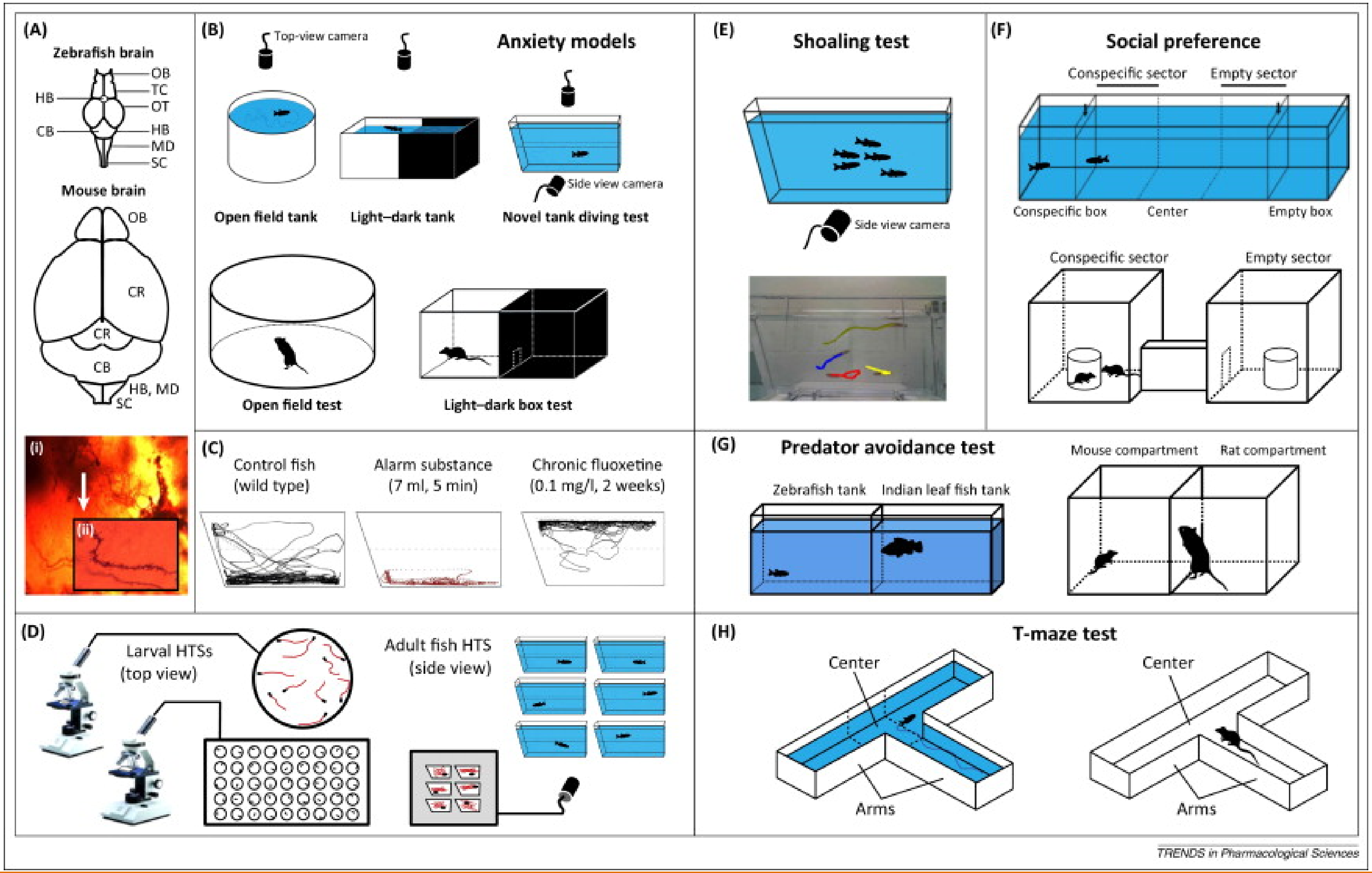
Added value zebrafish for DNT

- Complete and complex neuronal network (brain).
- Occurs within 3 days post-fertilization (dpf).
- Brain regions are well-conserved
- Neuronal subtypes well-conserved
- Zebrafish develops a blood brain barrier (BBB)
- Thyroid axis is present and affects brain development



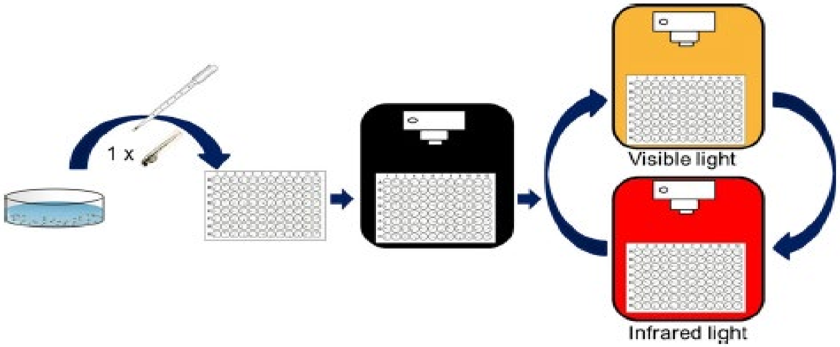
Kozol et al., 2016

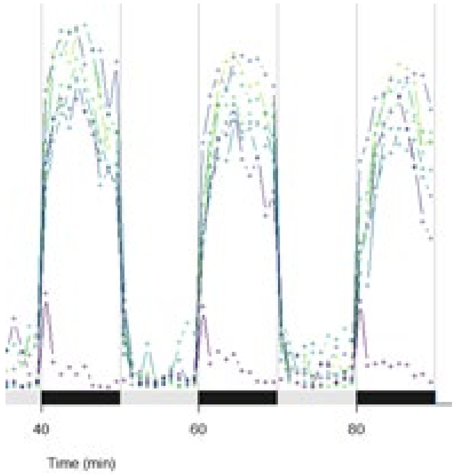
Examples DNT behavioral assays zebrafish



Light/dark transition (L/D) test

- Alternating light and dark periods
- 4 – 5 dpf
- Locomotor activity measured
  - Distance
  - Speed
  - Timing and area of movement
- Increased activity during the short dark phase and less activity during the light phase





Goals of this case study

- Current goal - determine the added value of the L/D zebrafish assay for DNT
  - Design a harmonized protocol
  - Determine inter-laboratory reproducibility by performing a case study in different laboratories
  - Discuss the results and protocol

- Next goal - determine the added value of the LD zebrafish test the guidance document / IATA

Institutes and people involved

Institute	People
U.S. Environmental Protection Agency	Bridgett Hill Stephanie Padilla Tim Shafer
Oregon State University	Lisa Truong Robyn Tanguay
Division of the National Toxicology Program, National Institute of Environmental Health Sciences	Jui-Hua Hsieh Kristen Ryan Mamta Behl
National Research Council of Canada	Lee Ellis
ZeClinics, Barcelona	Valentina Schiavone Davide Rubbini Javier Terriente
Biobide	Ainhoa Alzualde Arantza Muriana
VU University Amsterdam	Jessica Legradi
Health Canada / Government of Canada	Cindy Woodland
Organisation for Economic Co-operation and Development (OECD)	Magdalini Sachana
European Commission -DG Joint Research Centre	Anna Price
Helmholtz Centre for Environmental Research GmbH - UFZ, Germany	Nils Klüver
National Institute for Public Health and the Environment (RIVM)	Ellen Hessel

Material and Methods – Case Study

Phenotypic Evaluation

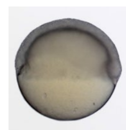
2 protocols:

FET - Fish Embryo Toxicity test (phase 1)
light-dark transition test (phase 2)

FET Assay
6 -120 hpf

Phenotypic
observations 120 hpf

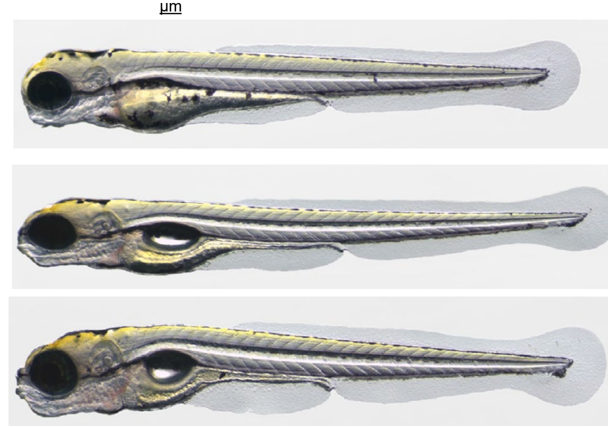
6 hpf



72 hpf

96 hpf

120 hpf



Phenotypic
observations 120 hpf

Behavioural 120 hpf
distance travelled in light dark “startle” response

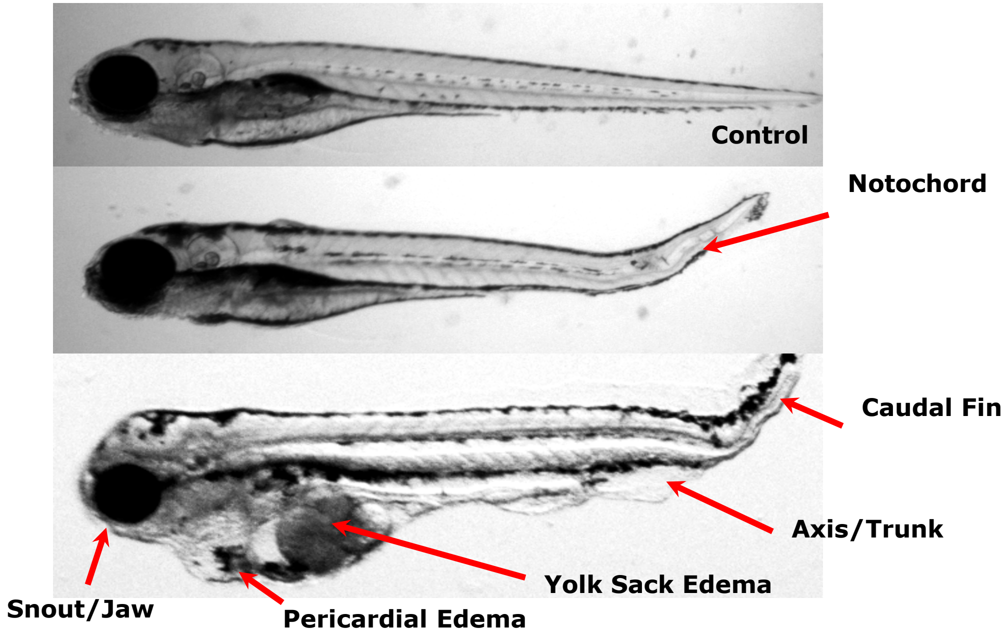
- Danio rerio AB strain
- 96 well forma round vs squared
- **Chorionated** embryos - FET
- Buffered media (10 mM pH7.2 HEPES E3 media)
- **Static** exposure
- 28°C, 14hr:10hr (light:dark)
- DMSO max. 0.4%

Images from Sol Gomez de la Torre Canny et al., Dev Dyn. 203:253-310. 2009.

Study design

DNT IATA CASE: Test Method for Zebrafish Inter-laboratory Validation

PHASE 1	PHASE 2	PHASE 3
Dose-range finding	Behavioral Assay: test and optimization	Definitive Study



FET – fish embryotoxicity test

0,1 – 3 – 100 uM

DNT IATA CASE: Test Method for Zebrafish Inter-laboratory Validation

PHASE 1 Dose-range finding	**PHASE 2** Behavioral Assay: test and optimization	PHASE 3 Definitive Study

Experimental Task	**Breeding**	**Drug Exposure**					**Malformation Evaluation & Behavioral Assay**
Hours Post fertilization		6-8	24	48	72	96	120

Critical Points

Well-plate

96 wells squared vs round
48 wells
group/concentration
Sealed vs Unsealed

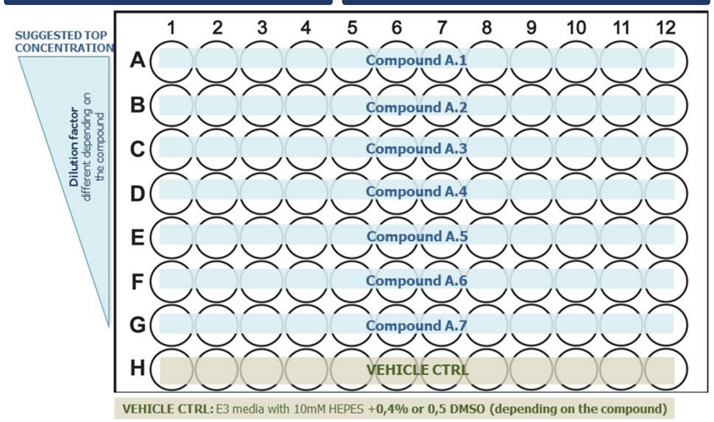
Exposure

Single dose
vs
Repeated dose

Light/Dark Transition Test

10’ on/10’ off; 10 min on; 20 min off
Some labs additional 40’on/40’off

96-well Plate Layout



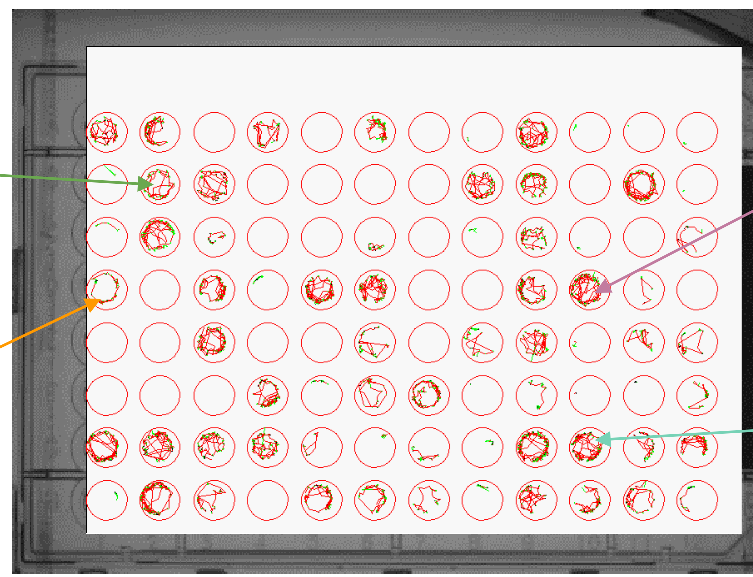
**Positive and Negative Controls on a separate plate*

Experimental Task	Breeding	Drug Exposure		Malformation Evaluation & Behavioral Assay
Hours Post fertilization		6-8	24, 48, 72, 96	120
Critical Points		**Well-plate** 96 wells squared vs round 48 wells group/concentration Sealed vs Unsealed	**Exposure** Single dose vs Repeated dose	**Light/Dark Transition Test** 10’ on/10’ off; 10 min on; 20 min off Some labs additional 40’on/40’off

Readout

Negative control

Hypoactivity



Positive control

Hyperactivity

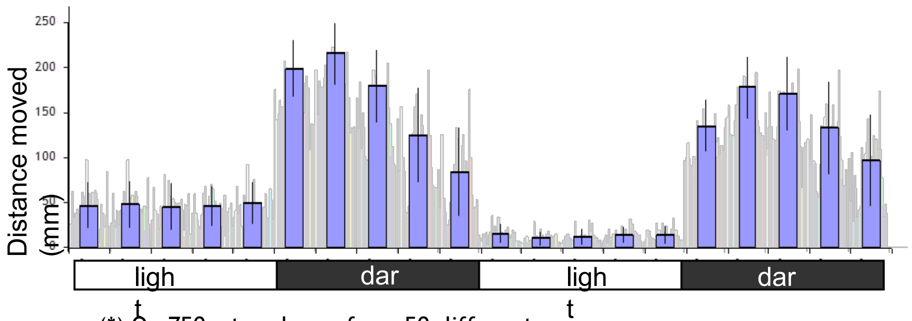
Protocol - 28 compounds

Chemical Name	CAS
3,3',5,5'-Tetrabromobisphenol A	79-94-7
Acetamiprid	135410-20-7
Allethrin	584-79-2
Benomyl	17804-35-2
Diazinon	333-41-5
Heptachlor	76-44-8
Nicotine	54-11-5
Parathion	56-38-2
Permethrin	52645-53-1
Trichlorfon	52-68-6
Triphenyl phosphates isopropylated	68937-41-7
Tris(1,3-dichloro-2-propyl) phosphate	13674-87-8
Tris(2-chloroethyl) phosphate	115-96-8
Tris(2-chloroisopropyl)phosphate	13674-84-5

Chemical Name	CAS
tert-Butylphenyl diphenyl phosphate	56803-37-3
2-Ethylhexyl diphenyl phosphate	1241-94-7
Aldicarb	116-06-3
Chloramben	133-90-4
Chlorpyrifos	2921-88-2
Cypermethrin	52315-07-8
Deltamethrin	52918-63-5
Dieldrin	60-57-1
Dimethoate	60-51-5
Kepone	143-50-0
Methyl parathion	298-00-0
Thiacloprid	111988-49-9
Tri-o-cresyl phosphate	78-30-8
Tris(methylphenyl) phosphate	1330-78-5

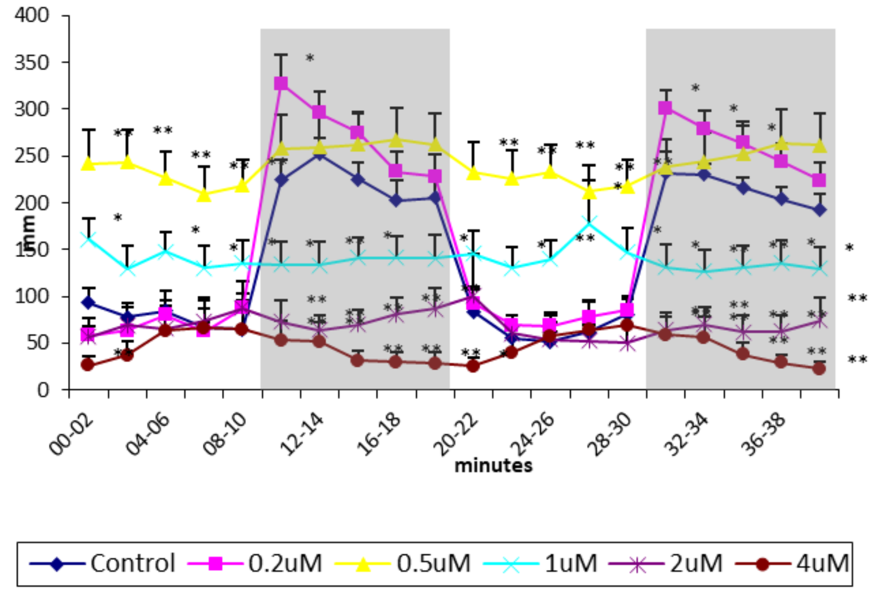
Example of the data

- DNT data
  - Distance moved (every minute)
  - Velocity (every minute)



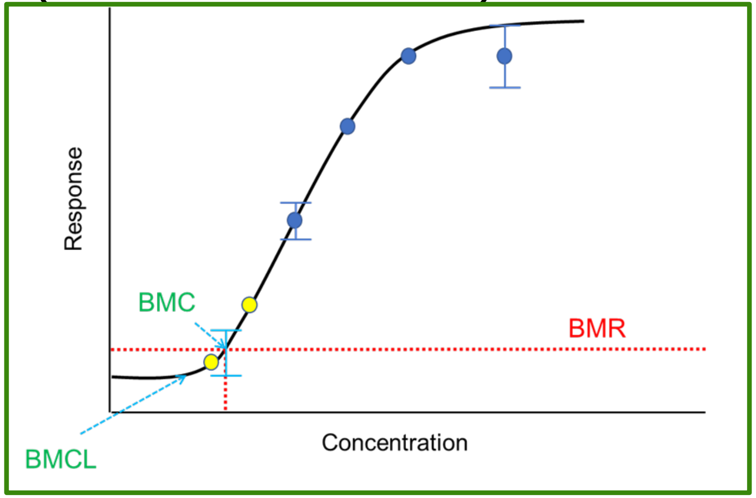
(*) On 750 wt embryos from 50 different plates

- Morphology data
  - Morphological Change/Dead observed
  - Swim_Bladder_Issue
  - Larvae included Y/N



Data analysis

- Application of benchmark dose (BMD) modeling
  - Focus on dose-response trend and onset of the response
  - Used in quantitative risk assessment
- Evaluate the <u>direction</u> and <u>amount</u> of movement during light/dark stimulation (vs vehicle control)
- Evaluate the similarity of the movement <u>pattern</u> across experiment (vs vehicle control)



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SOT | Society of Toxicology
www.toxsci.oxfordjournals.org

TOXICOLOGICAL SCIENCES, 167(1), 2019, 92–104

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Advance Access Publication Date: October 13, 2018
Research Article

Application of Benchmark Concentration (BMC) Analysis on Zebrafish Data: A New Perspective for Quantifying Toxicity in Alternative Animal Models

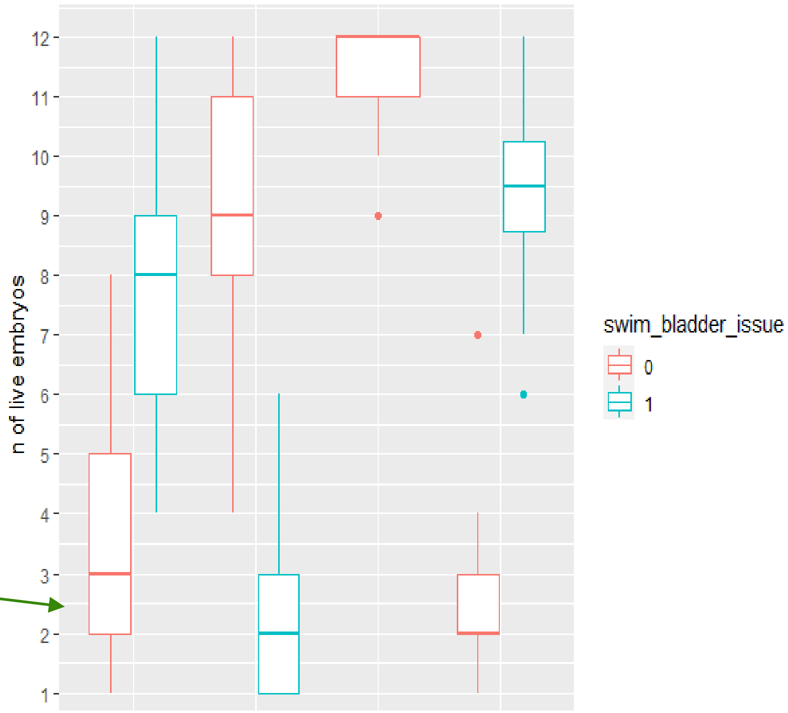
Jui-Hua Hsieh,[*,1] Kristen Ryan,[†] Alexander Sedykh,[‡] Ja-An Lin,[§] Andrew J. Shapiro,[†] Frederick Parham,[†] and Mamta Behl[†]

Median number of embryos without swim bladder issue varies between laboratories

The median number of embryo:
- normal swim bladder (0 - orange bars)
- uninflated swim bladder (1 - blue bars)

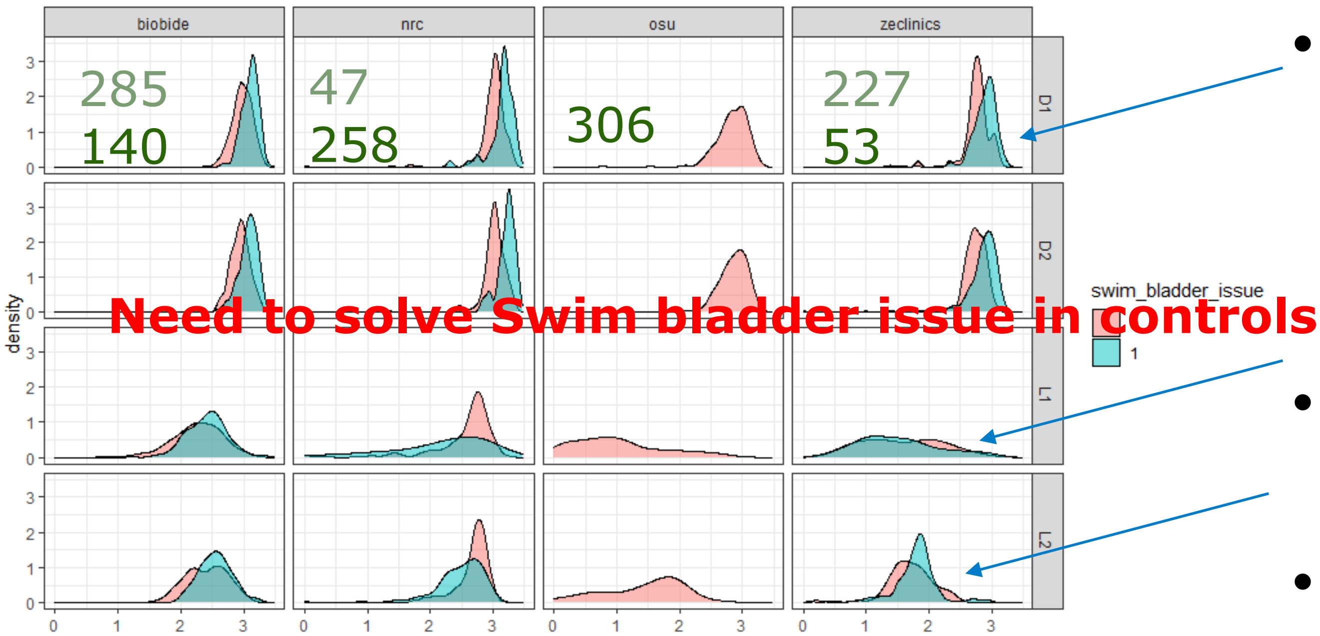
Lab1 (3/12), Lab2 (9/12), Lab3 (12/12),

Lab4 (2/12)



Round 96 well plate, 150 µl
Data could not be used!

Significant distance moved difference between embryos with and without swim bladder issue

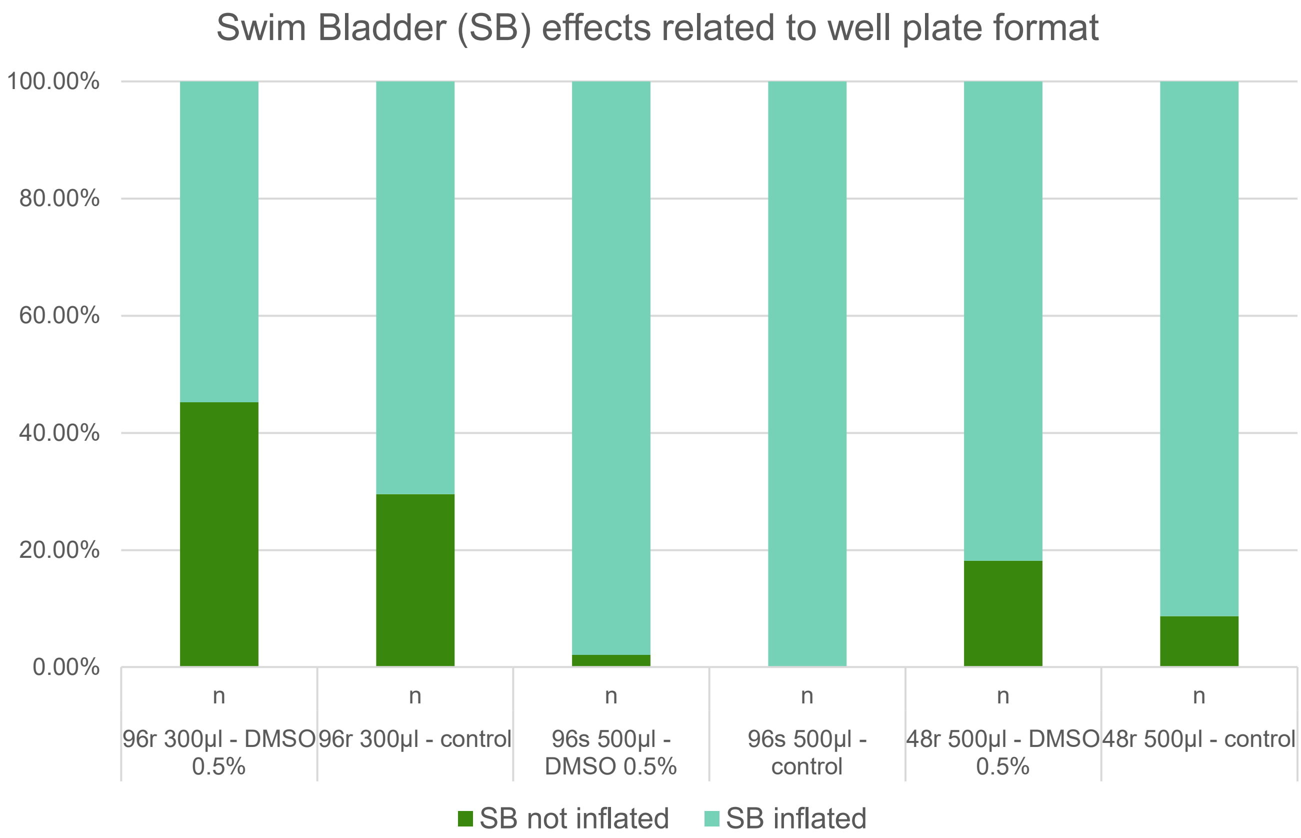


Distance moved

Round 96 well plate, 150 µl

- Mean shift to the right (larger movement), especially in D phases for embryos with swim bladder issue
- Larger movement variation in L phases
- Difference is significant (Wilcoxon test) except zeclinics:L1 and zeclinics:L2

Swim bladder issue affected by well volume

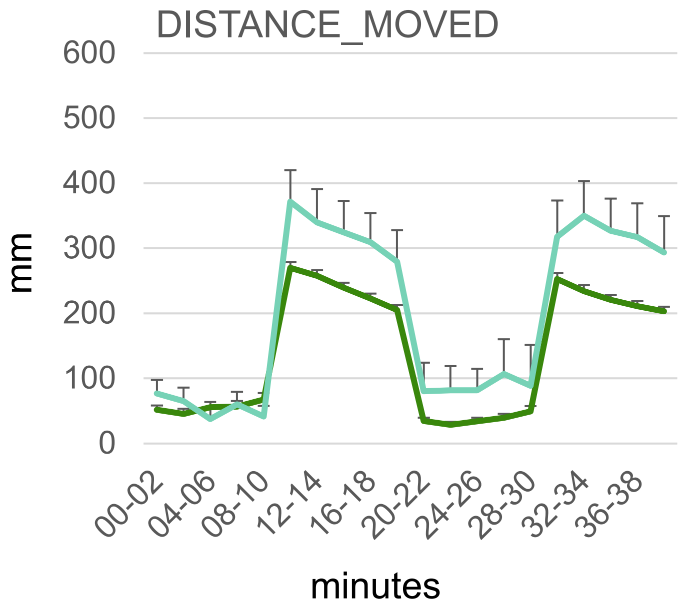


r = round
S= squared
Total 12 / group

EXPERIMENT #1

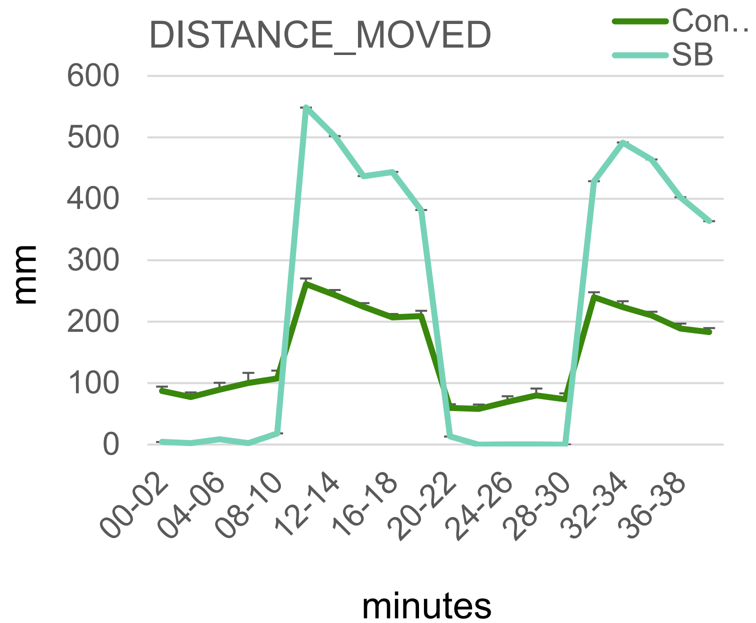
The plates were not sealed

Round 96 well plate – 300 uL



(Control) Swim bladder present:71
(SB) Swim bladder absent: 15

Square 96 well plate – 600 uL



(Control) Swim bladder present:74
(SB) Swim bladder absent: 1

Questions that will be answered? Protocol

Stability compounds

swim bladder

Experimental Task	Breeding	Drug Exposure					Malformation Evaluation & Behavioral Assay
Hours Post fertilization		6-8	24	48	72	96	120

Critical Points

Well-plate

96 wells squared round
48 wells
group/concentration
Sealed vs Unsealed

Exposure

Single dose
vs
Repeated dose

Light/Dark Transition Test

10' on/10' off; 10 min on; 20 min off
Some labs additional 40'on/40'off

Raising of the fish
- Well volume essential

Exposure period / time and dosing

Protocol light-dark period (time)

Questions that will be answered? Results

Discuss best protocol

Reproducibility among labs

Experimental Task	Breeding	Drug Exposure		Malformation Evaluation & Behavioral Assay
Hours Post fertilization		6-8	24, 48, 72, 96	120
Critical Points		**Well-plate** 96 wells squared vs round 48 wells group/concentration Sealed vs Unsealed	**Exposure** Single dose vs Repeated dose	**Light/Dark Transition Test** **10' on/10' off; 10 min on; 20 min off** **Some labs additional 40'on/40'off**

Comparable results with earlier studies.

Are known DNT compounds positive?

Take home messages pilot study

- Zebrafish behavior models can have an added value to the OECD guidance document for DNT.

- Volume in well plate affects swim bladder inflation

- Harmonization of the protocol is essential

- Inter-laboratory replication is a challenge

- Key players in the field are working together to develop a harmonized protocol for the light-dark transition test.

- In future protocols for other zebrafish behavioral tests for DNT can be harmonized.

Acknowledgements

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U.S. Environmental Protection Agency	Bridgett Hill Stephanie Padilla Tim Shafer
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