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Transitioning from mammalian animal testing to non-animal testing using the zebrafish (Brachydanio rerio) embryo as a whole organism model for regulatory decision-making in chemical risk assessments

Government of Canada

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What is Health Canada (HC)?

- Government of Canada department with broad mandate to promote and protect the health of Canadians

- New Substances Program* is responsible for assessing the risk of new substances to human health and the environment, jointly with Environment and Climate Change Canada, prior to approval to import or manufacture in Canada

- Chemical risk assessments (CRA) as required under the *Canadian Environmental Protection Act* (CEPA) 1999

- CRA = Hazard assessment + Exposure assessment =

  Potential Risk to human health and the environment

* https://www.canada.ca/en/environment-climate-change/services/canadian-environmental-protection-act-registry/general-information/fact-sheets/assessment-new-substances.html

HC risk assessments – Types of chemicals

Commodity Group	Examples
Personal care products • ***Cosmetics*** • ***Drugs (Over The Counter)*** • ***Natural Health Products***	surfactants, emulsifiers, UV blockers - *baby lotion, sunscreen, shampoo, toothpaste, shaving cream*
Industrial chemicals	flame retardants, plasticizers, surfactants
Food additives	flavour, sweetener, mineral fortifications
Human pharmaceuticals	active ingredients in medication
Veterinary pharmaceuticals	companion (pets) and livestock use

National Research Council (NRC) Canada

- Primary research and technology organization of the Government of Canada

- Zebrafish (ZF) research facility established in 2007
  - Expertise in ZF research and models
  - Develop ZF models
  - Validated standardized ZF assays
    - General toxicity evaluation
    - ADME (absorption, distribution, metabolism, elimination)
    - Behavioral evaluation (neurotoxicity [NT])
    - Developmental evaluation
    - Genomics facility – whole genome RNA sequencing

What is HC-NRC investigating?

In the context of the global shift away from animal testing:

- *Can the ZF embryo model serve as a robust New Approach Method (NAM) for predicting human toxicity in chemical risk assessments (CRA)?*

- *Can the ZF embryo model replace the rodent model to robustly predict chemical toxicity to humans and satisfy the 3 Rs of **R**educing, **R**efining and **R**eplacing animals in toxicity testing, at the same time?*

Comparison of ZF with rodent as a model

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

- High human relevance
  - ~70% gene concordance
- Cost is ≤1% of the cost for rodent studies
- Higher throughput
  - Many embryos + shorter life stages

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

- Embryos are transparent
  - ease of internal observations
- Greater statistical power

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

HC-NRC ZF Project

Refine ZF to be regulatory tool that transitions animal to non-animal testing

Refine ZF to enhance evaluations for:

- *General toxicity (GT) and behavior (GBT)*
- *Endocrine disruption (ED) (include more endocrine biology than EATS*)*
- *Transcriptomics (Tx) (evaluating effects on gene expression)*
- *ADME (absorption, distribution, metabolism and elimination)*

** EATS – estrogen, androgen, thyroid, steroidogenesis*

Images taken from:
http://conferences.genetics-gsa.org/zebrafish/index
https://www.labmanager.com/product-focus/2015/05/microplates-for-cell-based-assays

ZF International Collaborations

- NRC
  - Multi-year plan for collaborative ZF research projects

- NTP (U.S. National Toxicology Program)
  - SEAZIT (Systematic Evaluation of the Application of the Zebrafish in Toxicology)
  - SEAZIT inter-lab (4 labs) validation study
    - role of chorion in bioavailability
    - exposure paradigm (5-d static vs. daily renewal)
  - HC-NRC cross-validation with SEAZIT* inter-lab validation ZF study using 20 of the SEAZIT test substances

- OECD DNT (Developmental Neurotoxicity) WG
  - Developing standardized ZF protocol for DNT
  - Inter-lab validation for behavioral assessment – (inc. NTP)

https://ntp.niehs.nih.gov/whatwestudy/topics/dev-tox/seazit/index.html.

Objectives of HC-NRC ZF Research Project

1. *Refine embryo assay to enhance ED assessment (include more than EATS)*

2. *Refine larval (free swimming) assay to enhance GT assessment*

3. *Evaluate larval behavioral assay for predicting GT and NT*

4. *Include Tx evaluation (RNAseq) for predicting ED and GT*

5. *Use ADME to evaluate role of chorion in chemical bioavailability*

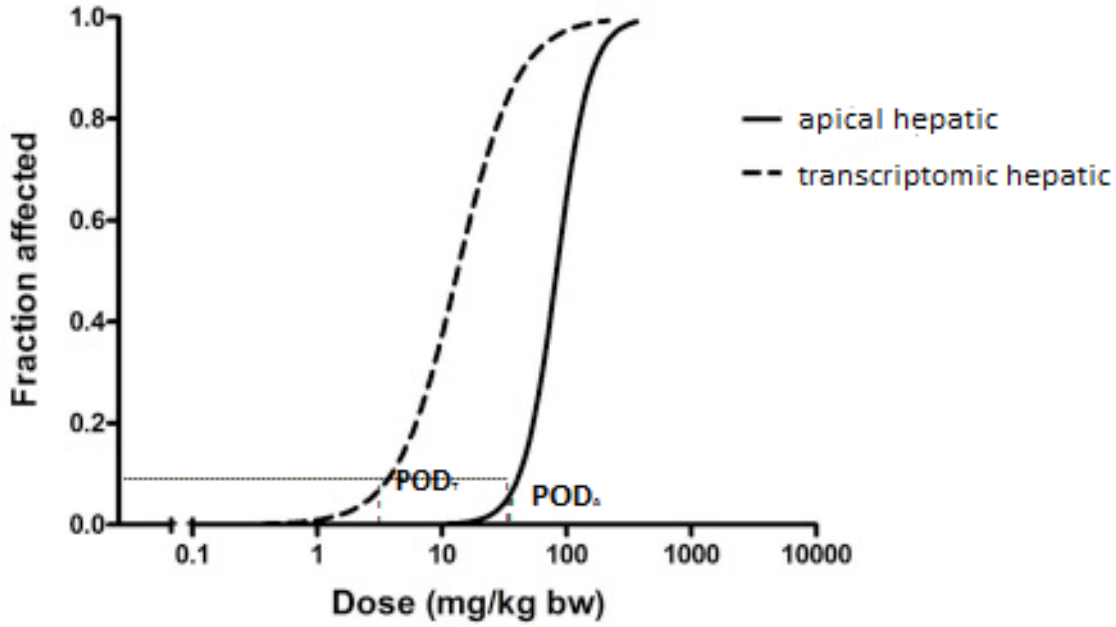
6. *Compare point of departure (POD) of Tx with observed phenotype POD*

   WHY? - Can Tx alone robustly predict human toxicity?

ED – Endocrine disruption
EATS – estrogen, androgen, thyroid, steroidogenesis
GT – General toxicity
NT – Neurotoxicity
Tx – Transcriptomics
ADME – absorption, distribution, metabolism, elimination

Is the transcriptomic (Tx) POD more sensitive than phenotypic (Ph) POD?

Theoretical representation of the POD from Tx vs Ph



POD_{Tx} may be more sensitive than POD_{Ph} for evaluating dose response

How are the ED and GT assays refined?

- Include Transcriptomics evaluation
- Include phenotypic endocrine endpoints that are in addition to conventional EATS markers, eg. obesogenicity and adrenal markers
- Correlate Tx gene expression with phenotypic findings

ZF study Test Substances

Name	CAS No.	List	Use
Dechlorane Plus	13560-89-9	NTP*	Replacement Flame Retardant
Bisphenol S	80-09-1	NTP*	Replacement Plasticizer
Triphenyl Phosphate (TPhP)	119-61-0	NTP*	Replacement Plasticizer and Flame Retardant
Tricresyl Phosphate (TCrP)	1330-78-5	NTP*	Replacement Flame Retardant
Tris(dichloro-isopropyl) phosphate (TDCPP)	13674-87-8	NTP*	Replacement Flame Retardant
Raloxifene HCL	82640-04-8	ICL, NTP*	Treatment of osteoporosis and breast cancer
Testosterone propionate	57-85-2	ICL, NTP*	Anabolic steroid, treatment of breast cancer
Permethrin	52645-53-1	ICL, NTP*	Human and veterinary insecticide (head lice and scabies)
Thiabendazole	148-79-8	ICL, NDSL, NTP*	veterinary fungicide
Benzophenone	119-61-0	NTP*	UV blocker, flavour ingredient, fragrance enhancer
Bisphenol A	80-05-7	NTP*	Plasticizer
Valproic Acid	99-66-1	NTP*	Anticonvulsant
Aldicarb	116-06-3	NTP*	Pesticide
Amoxicillin	26787-78-0	NTP*	Antibiotic
Pyrene	129-00-0	NTP*	Precursor for dyes, plastics and pesticides
Resorcinol	108-46-3	NTP*	Topical pharmaceutical for treatment of skin disorders
Pyriproxyfen	95737-68-1	NTP*	Veterinary drug for flea control
TBBPA	79-94-7	NTP*	Brominated flame retardant
Propofol	2078-54-8	NTP*	Pharmaceutical sedative
3,4-dichloroanilene	95-76-1	NTP*	Positive control (herbicide precursor)

NTP* = List of chemicals selected for inter-laboratory validation study of zebrafish embryo test

Regulatory Context

Can the zebrafish model serve as a robust *in vivo* regulatory tool that meets the 3Rs and predicts human health hazard as reliably or better than conventional *in vivo* rodent assays?

Acronyms

ADME	Absorption, Distribution, Metabolism and Excretion
CRA	Chemical Risk Assessment
DNT	Developmental Neurotoxicity
EATS	Estrogen, Androgen, Thyroid, Steroidogenesis
ECCC	Environment Canada and Climate Change
ED	Endocrine Disruption
GBT	General Behavioral Toxicity assay
GT	General Toxicity
HC	Health Canada
HT	Human Toxicity
HpF	Hours post-fertilization
NRC	National Research Council of Canada
NSP	New Substances Program
NT	Neuro-toxicity
NTP	[US] National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
POD	Point of Departure
SEAZIT	Systematic Evaluation of the Application of the Zebrafish in Toxicology
Tx	Transcriptomics

Study Design

2018-2019 Phenotypic Evaluation

NRC protocols:

FET - Fish Embryo Toxicity test - Endocrine disruption

GBT - General Behavior and Toxicity assay

3 replicates of n=12
7 dose levels + 0.5-1% DMSO control

FET Assay
- 6-120 hpf
Phenotypic
- observations 72,120 hpf
as dead or affected

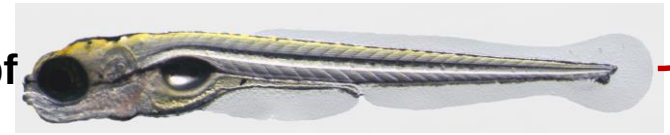
6 hpf

250 µm

72 hpf

96 hpf

120 hpf



GBT Assay
- 72-120 hpf
Phenotypic
- observations 96,120 hpf
as dead or affected
Behavioral 120 hpf
- distance travelled in light
- dark “startle” response

- 96 well format: 1 embryo in 300 µL media per well
- Chorionated - FET
- Buffered media (10 mM pH7.2 HEPES E3 media)
- **Static** exposure
- 28.5C, 14hr:10hr (light:dark)

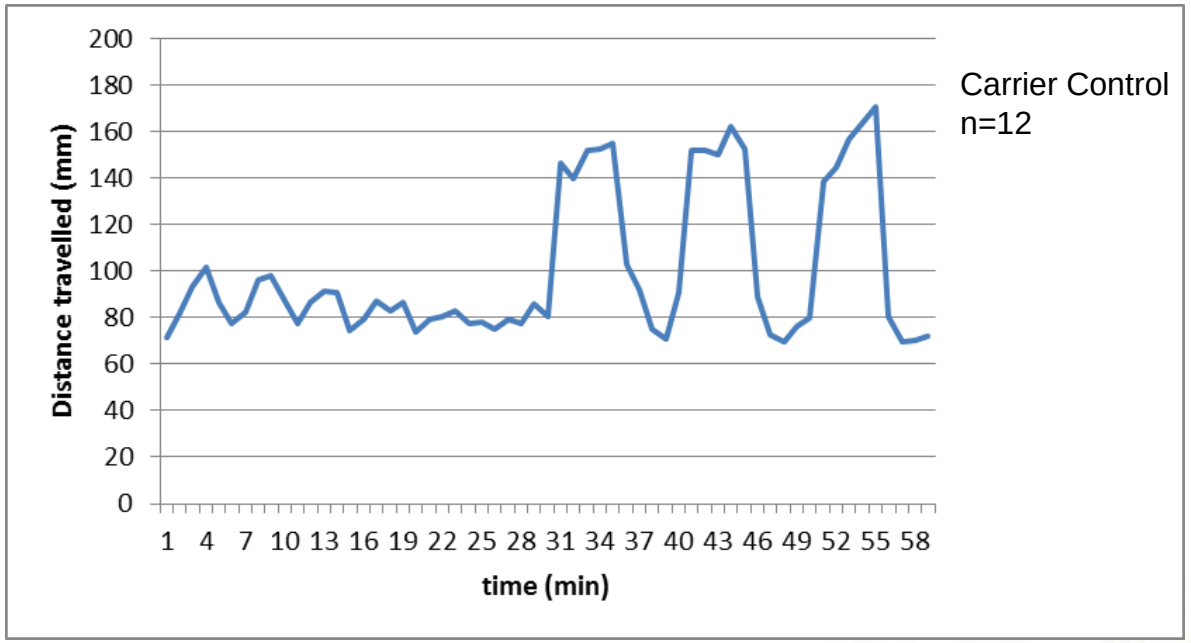
Images from Sol Gomez de la Torre Canny et al., Dev Dyn. 203:253-310. 2009.

Phenotypic Evaluation for ED and GT

Scoring acronyms

NOE: No observable effect (embryos phenotypically normal for stage, pigment @48hpf, hatched@72hpf)
H: Hatched
UH: Unhatched
LR: loss of lateral recumbancy (after 72hpf)
TSU: Trouble staying upright (after 72hpf)
PCE: Pericardiac edema
HB: Heart beat (eg. No: -, Slow)
BF: Blood flow
LC: Lighter colour
YSE: Yolk surface edema
MA: Melanocyte aggregation
DM: “Donut melanocyte”, pigment at outer edges of melanocytes
CM: constant movement
Nec: Necrotic/dead
Unf: embryo appears unfertilized/dead
GH: Grey(cloudy) head
ST: Stubby tail
SH: Small head
BP: Blood pooling
L: L-shaped body
C: C-shaped tail
J: J-shaped tail
Cu: Curled body (as if still in chorions)

NRC General Behaviour and Toxicity (GBT) Assay



Phenotypes scored: 96 &120 hpf

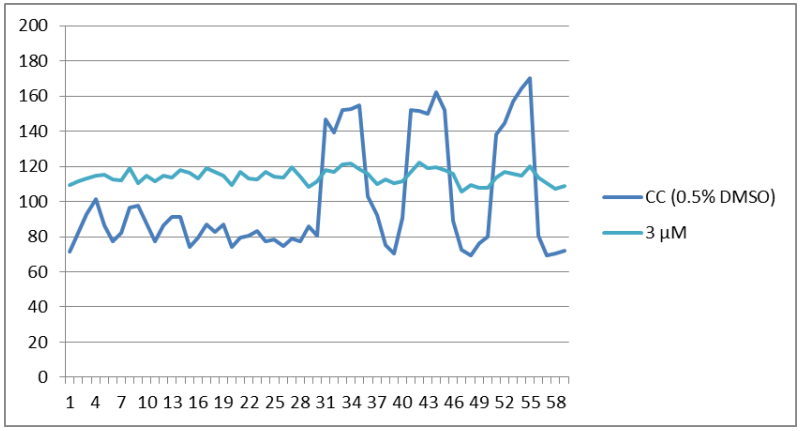
Behavioural Assay: 120 hpf

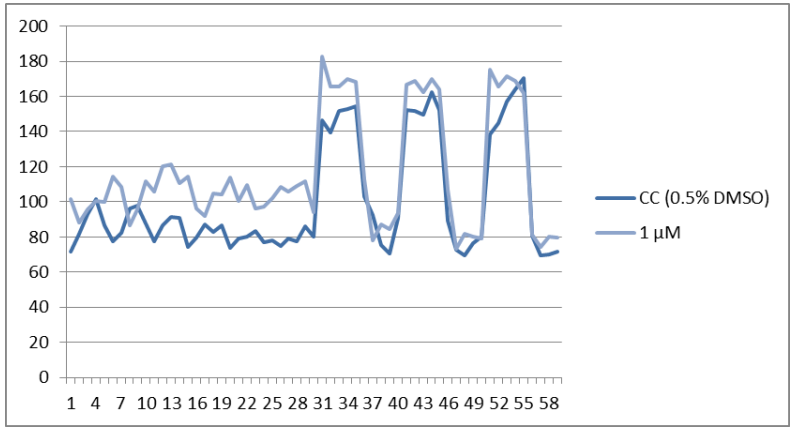
Light

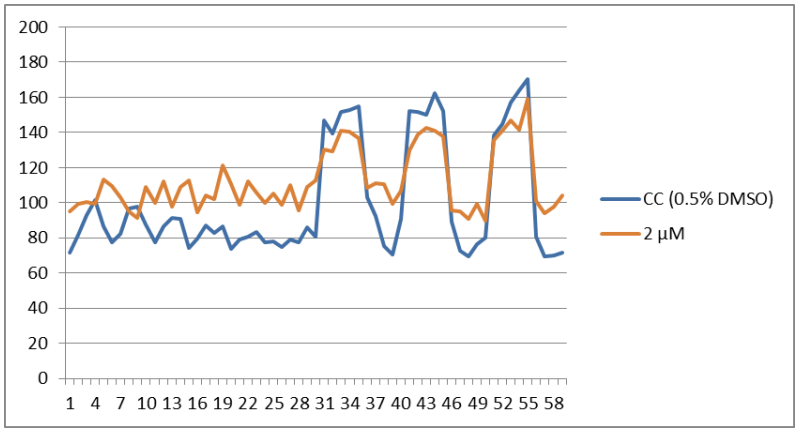
Dark

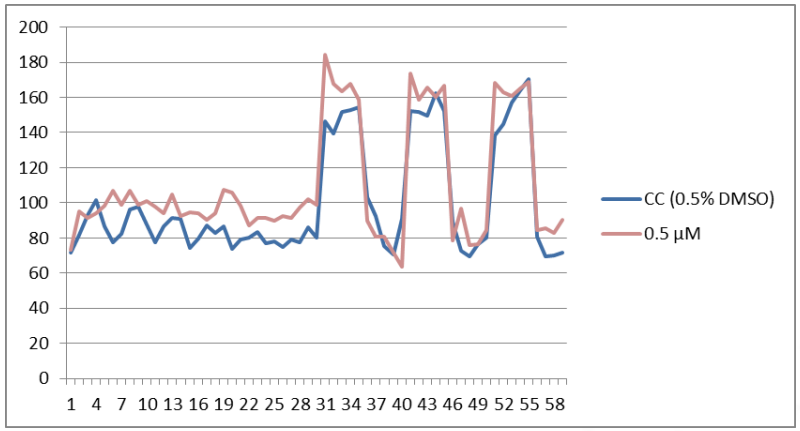
Quantifiable behavioural measurement

GBT Assay: Permethrin









- “Constant movement” phenotype
- Quantifiable behavioural measurement (n=36)

2019-2020: Tx and ADME

Transcriptomics Objective:

Correlate phenotypic results with Tx gene expression to determine ability of Tx to predict human toxicity.

ADME Objective:

Generate data on chorionic bioavailability and contribute these findings to the global regulatory discussion on the role of the zf chorion in CRA.

Transcriptomics (Tx)

Whole transcriptome generated using RNAseq

2018-19

- 3 chemicals (BPA, BPS, permethrin). Doses: EC_{20}, EC_{20} x 10^{-2}, EC_{20} x 10^{-3}

2019-20

- 8/20 chemicals (TDCPP, Testosterone propionate, thiobendazole completed)
- Revised dose regimen to include 5 doses to better fit dose curve:
  - eg. EC_{20} + 4 lower doses or $5EC_{20}$, EC_{20} + 3 lower doses
- Individual chemical dose selection based on potency (eg. high dose = EC_{20} or $5EC_{20}$)
- Doses lower than EC_{20} are10-fold dilutions ($EC_{20}x10^{-1}$, $EC_{20}x10^{-2}$, $EC_{20}x10^{-3}$, $EC_{20}x10^{-4}$)

- Development of Tx analysis ongoing
  - Whole genome / pathway analysis and possibly targeted gene analysis

- ED transcriptomic platform to build on HC and EPA obesogenicity research
  - eg. *ppar gamma* and *fa11a* genes (eg. phenotype: fat deposition around the swim bladder)

ADME Protocol

- 10/20 compounds
- Evaluate uptake at bath concentration of EC_{20}
- Sampling time points

  FET - 6, 8, 24, 48, 72, 120 hpf (ongoing)

  GBT - 72, 74, 76, 78, 96, 120 hpf (future)
- Analysis of bath and larval tissue

  - parent chemical + identify metabolites

Next Steps

- Transcriptomics
  - Conduct Tx for remaining 12/20 compounds
  - Develop analysis – pathway analysis

- ADME
  - Conduct ADME for remaining 10/20 compounds
  - Analysis to determine if chemicals pass through chorion

- Threshold for molecular weight permeability through chorion
- Correlate phenotypic data with Tx and ADME
- Is Tx POD more sensitive than Ph POD?
- Compare ZF data with existing rodent data

Thank you!

Questions?