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Larval Zebrafish Neurodevelopmental Toxicity Testing and Variables That May Affect the Outcome

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Disclaimer: The views expressed in this presentation are those of the author's and do not necessarily reflect the views or policies of the U.S. EPA

Office of Research and Development
Center for Computational Toxicology and Exposure, BCTD, RADB

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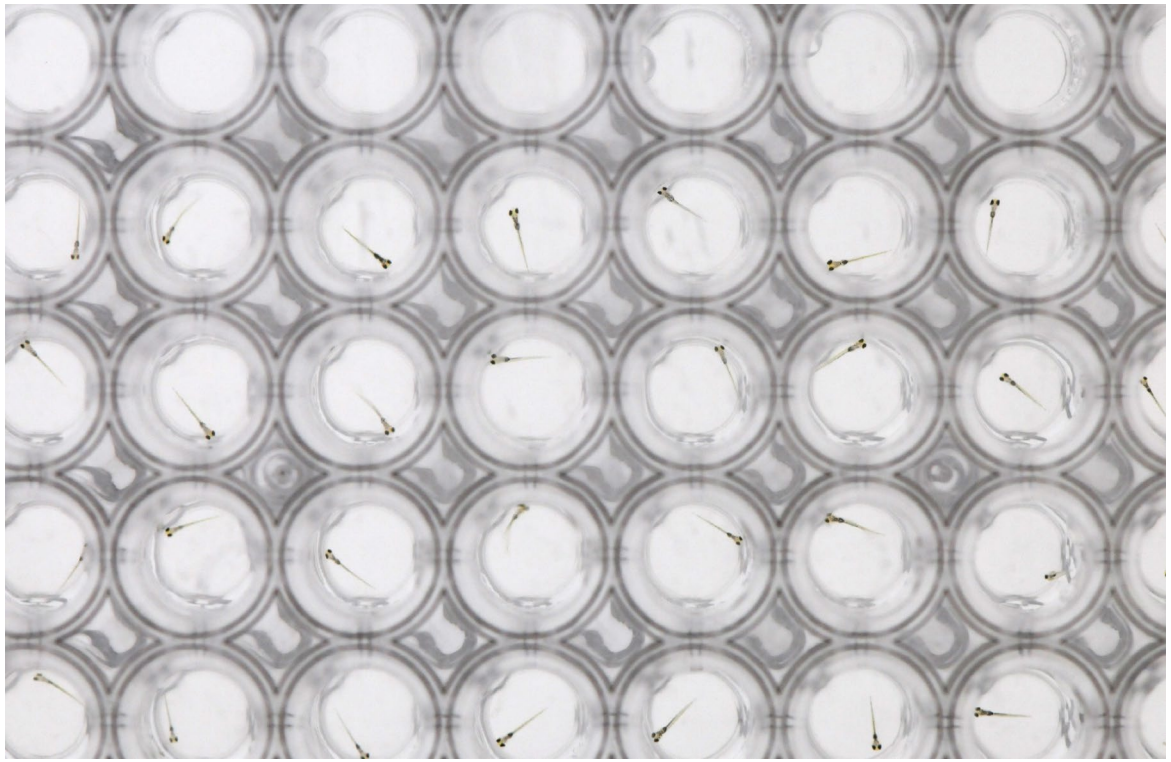
Larval Zebrafish Behavioral Tests:

- Inform us about the FUNCTION of an animal
- Inform us about how the animal responds to its environment
- Are not an endpoint in many other *in vitro* screens
- Are amenable to medium to high-throughput screening

Our own laboratory at the EPA is using these tests to screen chemicals for Developmental Neurotoxicity potential



However....

“Behavioral screens require vigilance in maintaining constant environmental conditions during raising and testing larvae, as seemingly trivial deviations can introduce unanticipated quantitative changes in responses.”

Harold A. Burgess and Michael Granato, The neurogenic frontier—lessons from misbehaving zebrafish, *Briefings in Functional Genomics and Proteomics*, vol. 7, no. 6, 474-482, 2008

Many research groups are trying to develop consistent protocols for zebrafish behavioral testing

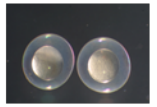
What is the basic structure of a zebrafish behavioral assay?

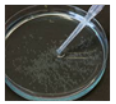
and what are some variables that may affect the results?

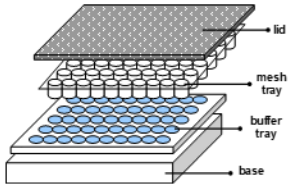
- Age of larvae
- Time of Day
- Malformation status
- Level of light during testing
- Presence of DMSO (dimethyl sulfoxide)
- Swim bladder inflation status
- Presence of test chemical

Exposure and Assessment

Day 0





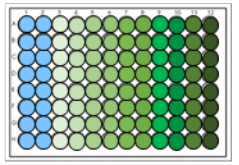


Spawning & Embryo Collection
- *0-0.5 hours post fertilization*
- *Embryos collected 30 minutes post-fertilization*
- *Beakers with embryos placed in 26˚C water bath for 2 hours*

Embryos Prepared
- *2-3 hours post fertilization*
- *Followed by embryo wash in 0.06% bleach solution*
- *Nonoptimal embryos removed from dish*

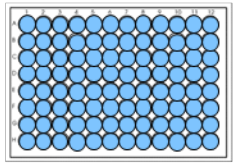
Embryos Plated
- *6-8 hours post fertilization*
- *96 well mesh microtiter plate, 1 embryo per well*
- *10% Hanks' solution or EPA Moderately Hard Water*

Day 3



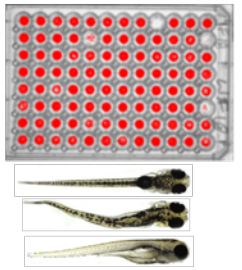
Test Chemical Solution Renewed
- *Continuation of day 0 Test Chemical Protocol*
- *61 Test Chemicals*
- *Control = 0.4% DMSO or Deionized Water*
- *Positive control = Chlorpyrifos (0.3, 1, 3 μM)*
- *Reared at 26°C, 14:10 light/dark cycle (lights on at 0730 h)*

Days 5-6



All Test Chemicals Removed
- *Larvae removed from Test Chemical solution*
- *No Test Chemicals*
- *10% Hanks' or EPA Moderately Hard Water only*

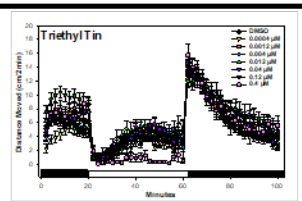
Day 6



Behavioral Testing
- *Renewed with 10% Hanks' or EPA Moderately Hard Water*
- *Acclimate in darkroom for at least 2 hours*
- *Testing Paradigm (Noldus Tower System and DanioVision)*
  - *20 minutes dark (basal period)*
  - *40 minute light (18 lux)*
  - *40 minute dark (0.5 lux)*

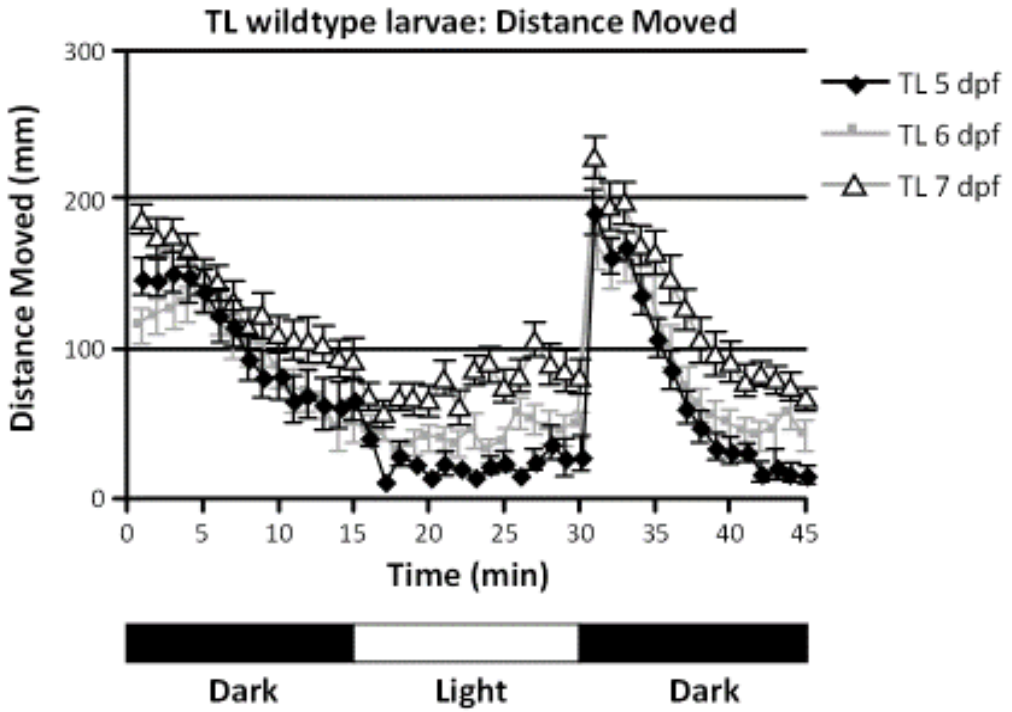
Lethality and Malformation Assessment
- *Immediately following behavioral testing*
- *Normal larvae identified*

Post-Assay Analyses

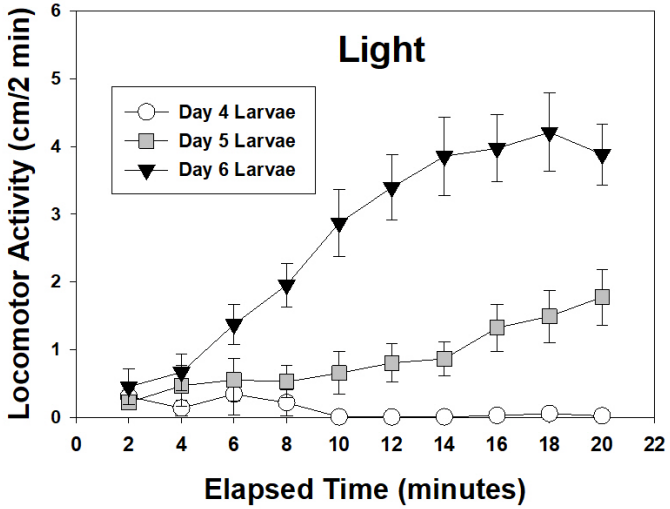


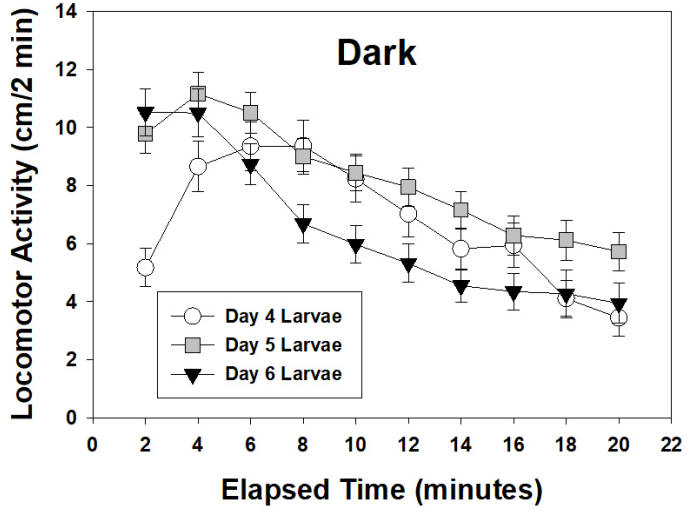
Locomotor Tracking & Data Analysis
- *Larval movement tracked (Ethovision software Version 13)*
- *Tracking rate = 5 samples/second*
- *Activity calculated for distance (centimeters) moved every 2 minutes (cm/2 min)*
- *Dead, malformed, unhatched & uninflated swim bladder removed from behavioral analysis*

Multiple studies in multiple laboratories have demonstrated that the age of the larva affects the light/dark behavioral profile



C de Esch *et al,* 2012.



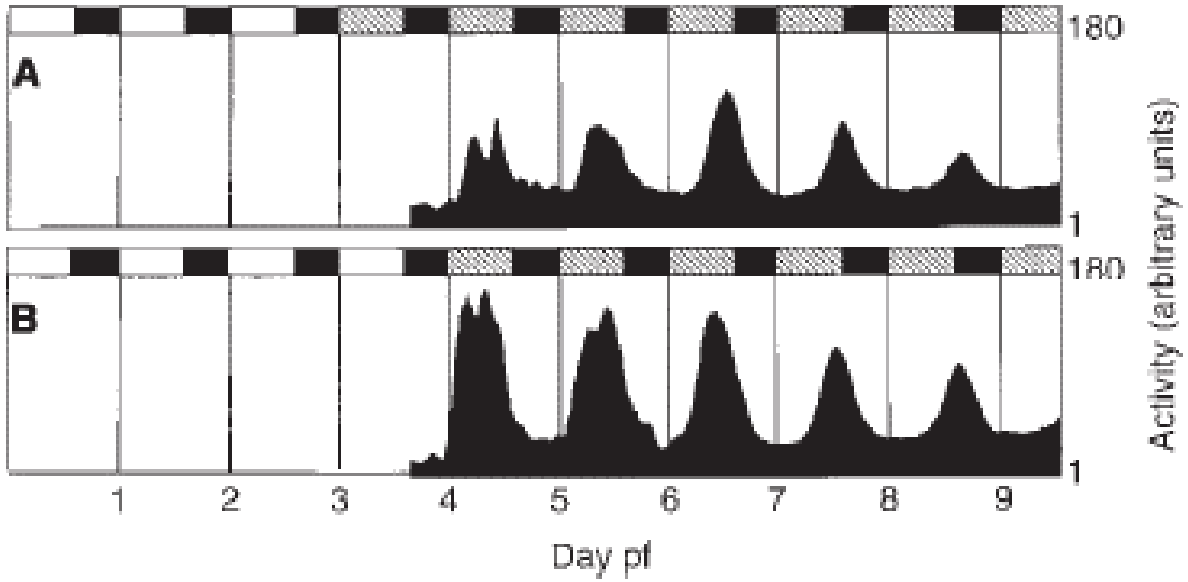


Padilla *et al*, 2011

Note consistent result of increasing activity in the light phase as the larva matures

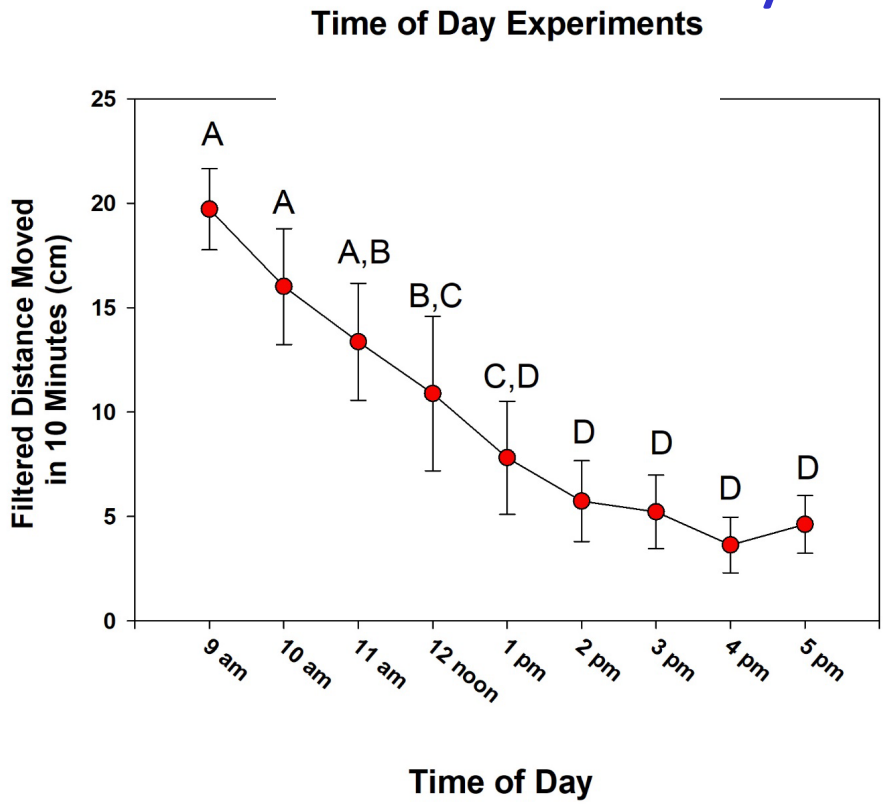
What time of day to test?

- Zebrafish larvae are very attuned to the time of day
- Used to study clock genes and melatonin surges



Hurd and Cahill, 2002

What time of day to test?



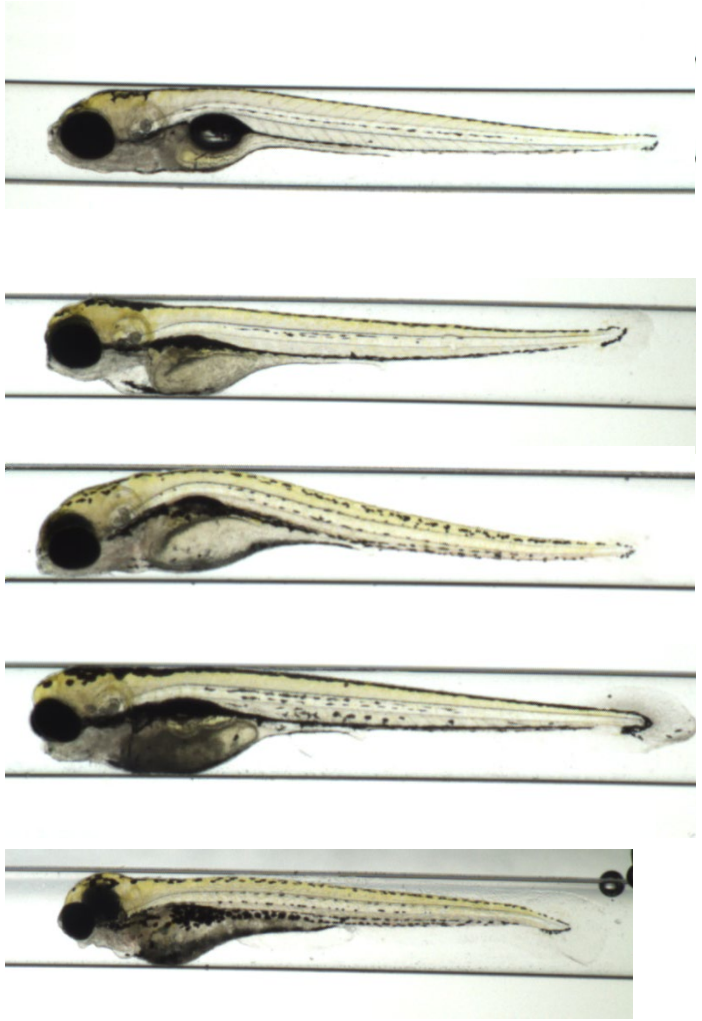
Time points with the same letter designation are not different from one another

- Baseline activity is high in the morning, decreasing toward noon
- Levels off in the afternoon
- Absolutely essential to have all experimental conditions on every plate to account for differences in baseline activity
- Best case scenario would be to test during the stable baseline activity approximately 5 hours after lights come on in the incubator

Intersection of Malformation and Behavior

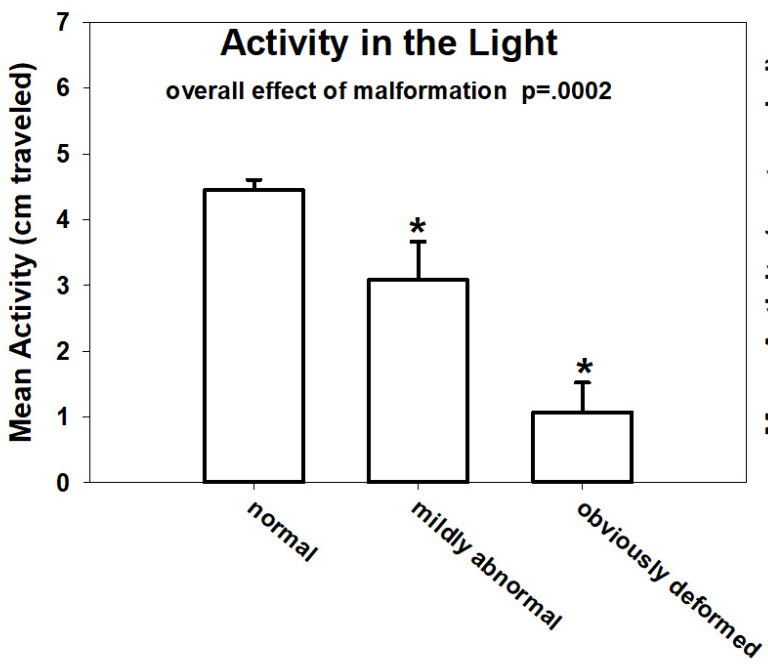
- Embryonic/larval development is complicated
  - Many things can go wrong
    - Some can be random
    - Some can be due to chemical treatment

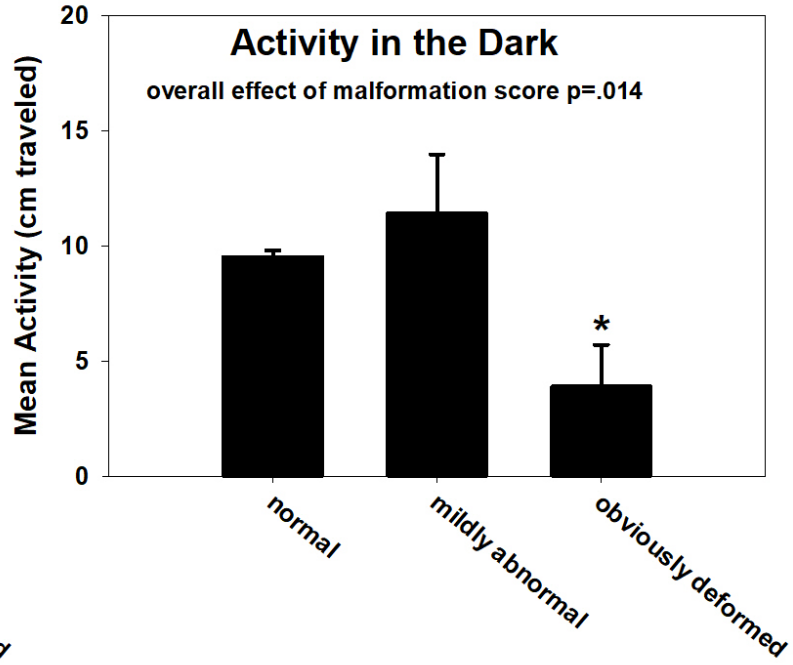
When behavior is the endpoint and developmental neurotoxicity is the question, it is important to remove malformed fish from the behavioral analysis as they may not be able to move like normal fish



These zebrafish pictures were taken using the VAST BioImager™ from Union Biometrica

Presence of Malformations Affects Behavior



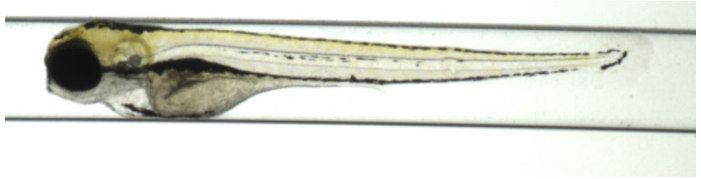


Padilla *et al*, 2011

Examples

Normal:

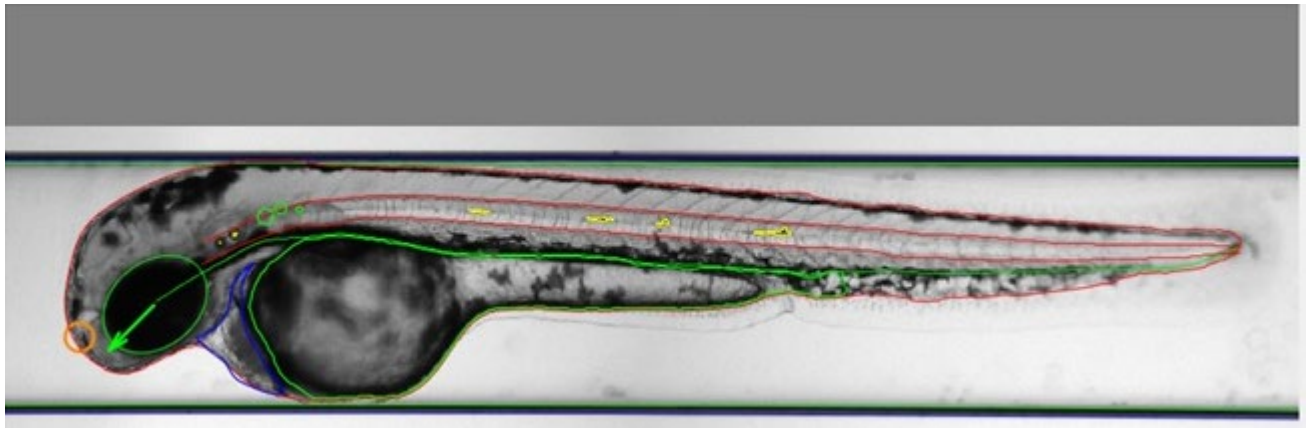
Mildly Abnormal



Obviously Deformed

Future of the Intersection of Structure and Behavior

- As our ability to catalogue, quantify and define the range of what is “normal” body structure for a zebrafish, the relationship between normal body structure and behavior will become clearer

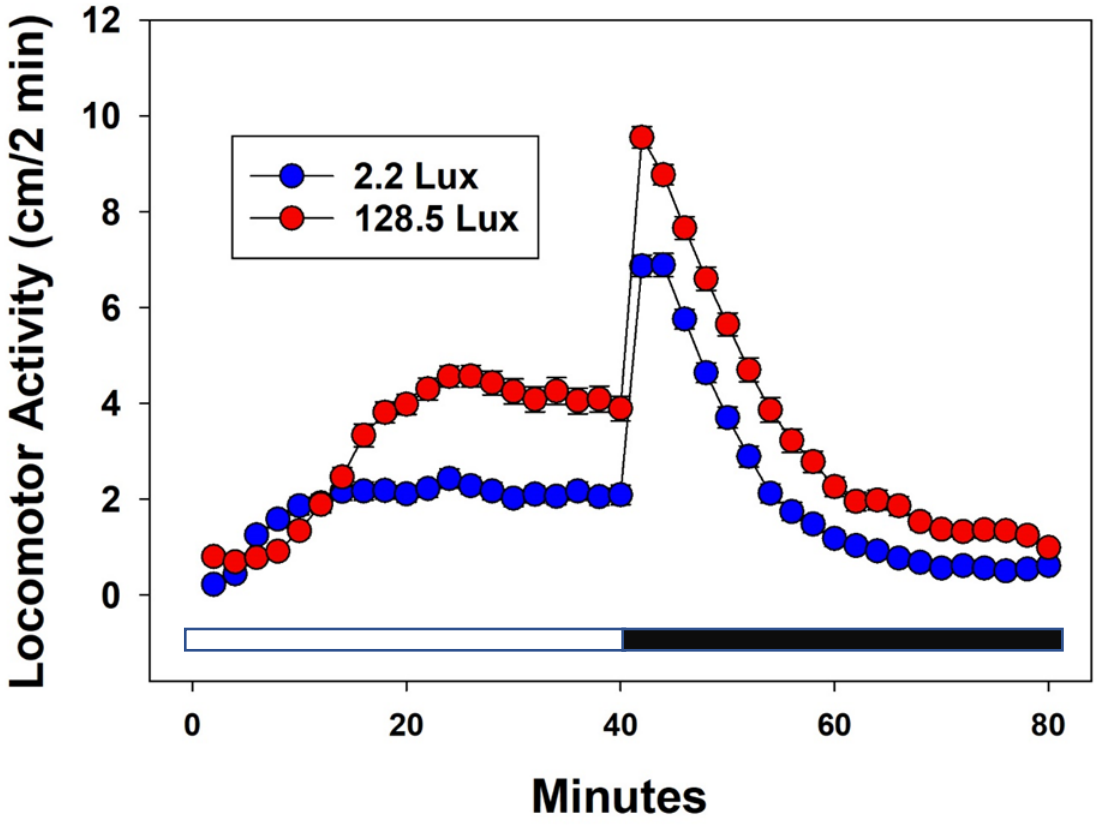


FishInspector; Helmholtz Centre for Environmental Research; https://www.ufz.de/index.php?en=44460

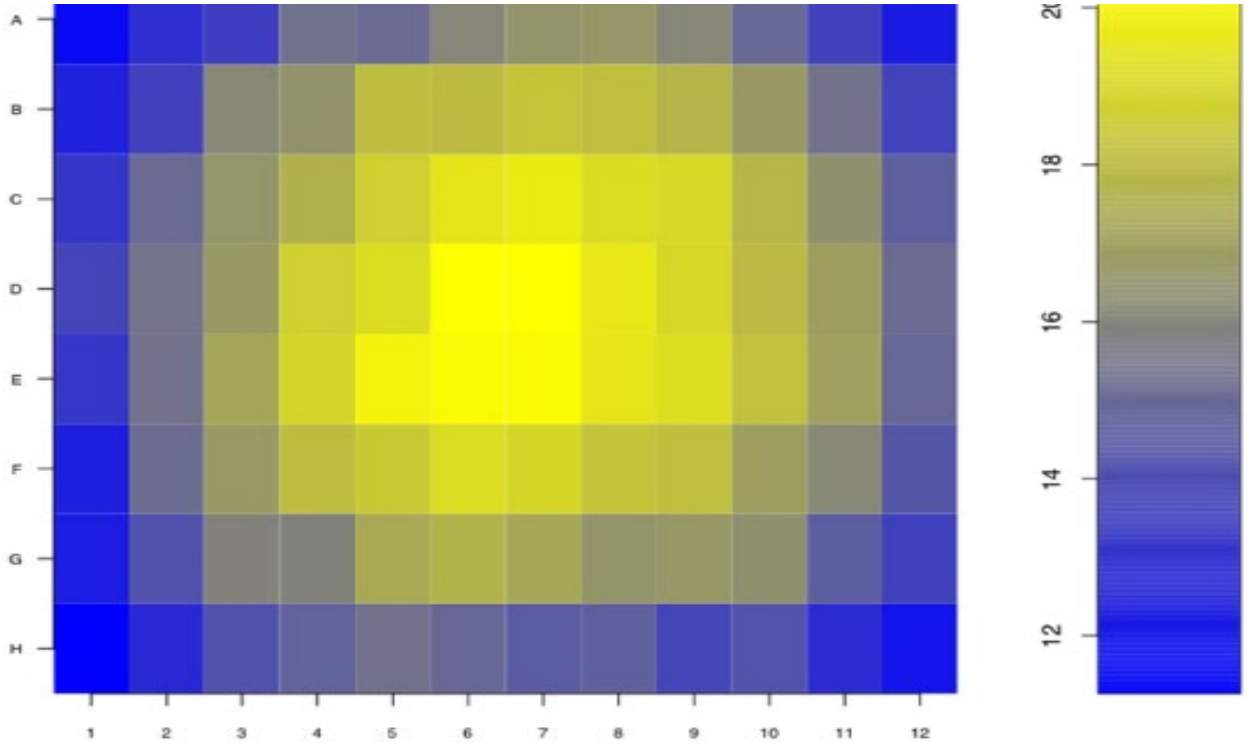
Light and Vision are Integral to Zebrafish Survival

- Zebrafish are highly visual animals
- They must be able to escape, hide and eat soon after hatching
- Zebrafish have four types of cone photoreceptors
  - Long wavelength sensitive (red)
  - Medium wavelength sensitive (green)
  - Short wavelength sensitive (blue)
  - UV wavelength sensitive (UV)
- Humans only have 3 types of cone photoreceptors
  - Difficult for humans to appreciate what fish are seeing

Different levels of light = Different Behavioral Profiles



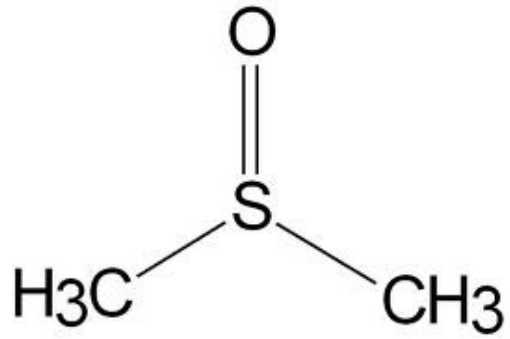
Light Level is variable across microtiter plate



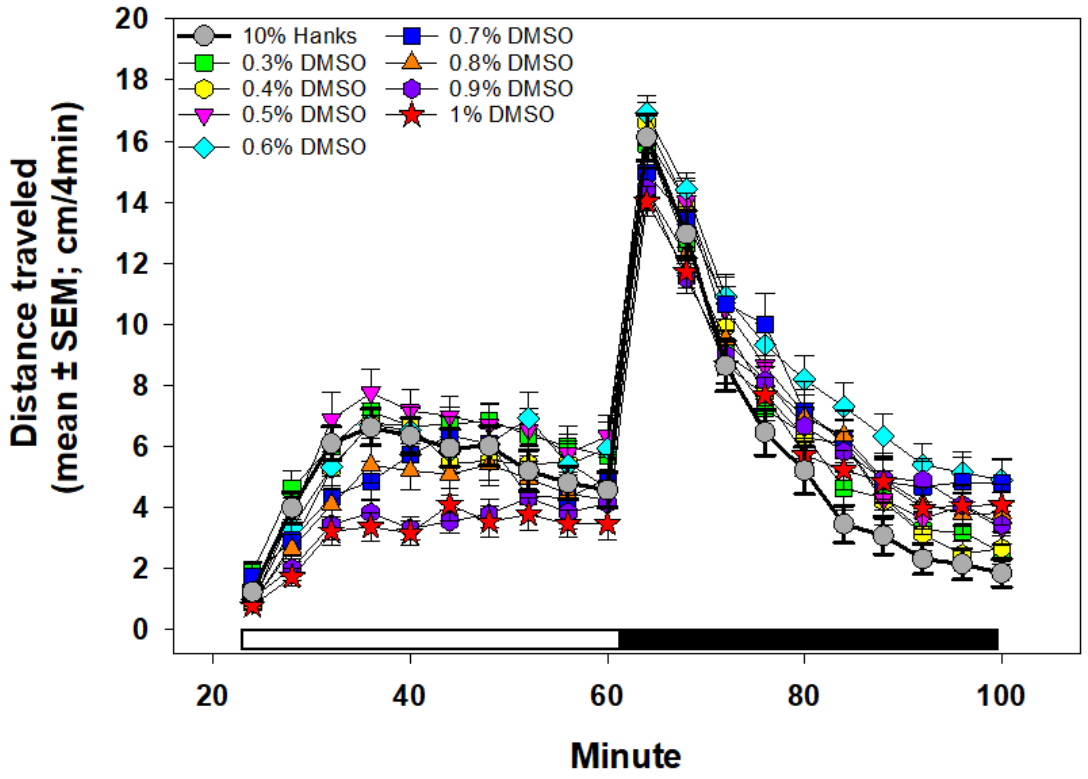
Xie et al, 2019 *PlosOne*

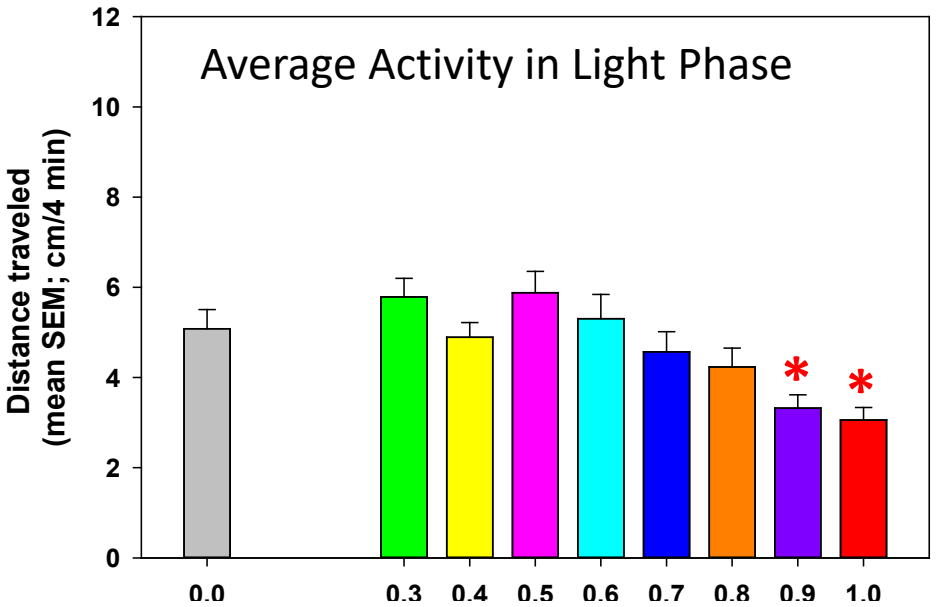
Dimethyl Sulfoxide (DMSO)

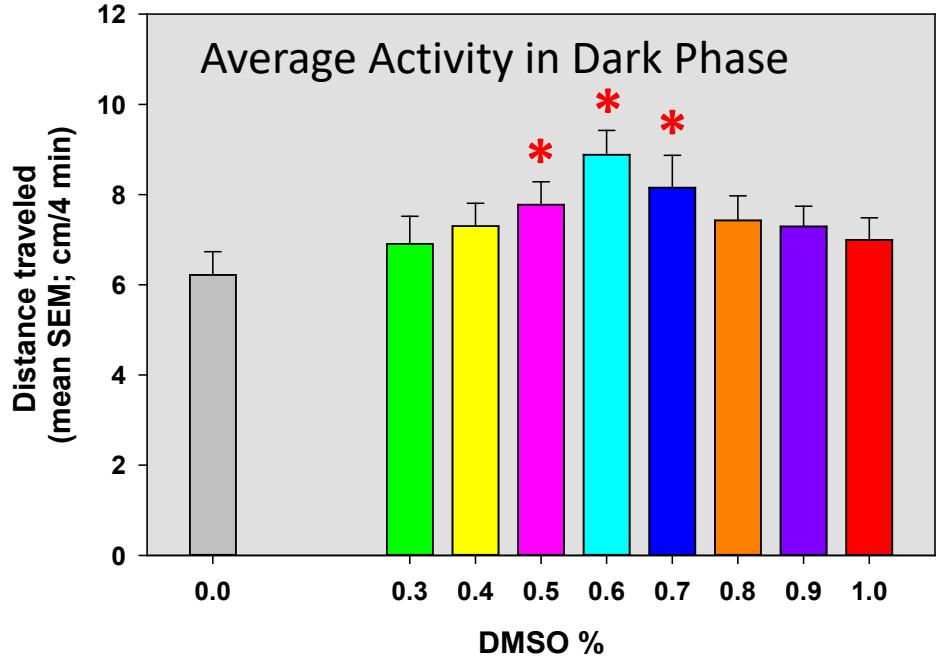
- Often used as a vehicle for chemical screening
- “Universal” solvent
- Convenient to use as a vehicle
- DMSO is developmentally toxic to zebrafish above 1% (v/v)
- Previous reports that DMSO may affect larval zebrafish behavior, but lack consistent results



Exposure to DMSO during development decreases activity in the light and increases activity in the dark. Safe range is ≤ 0.4%





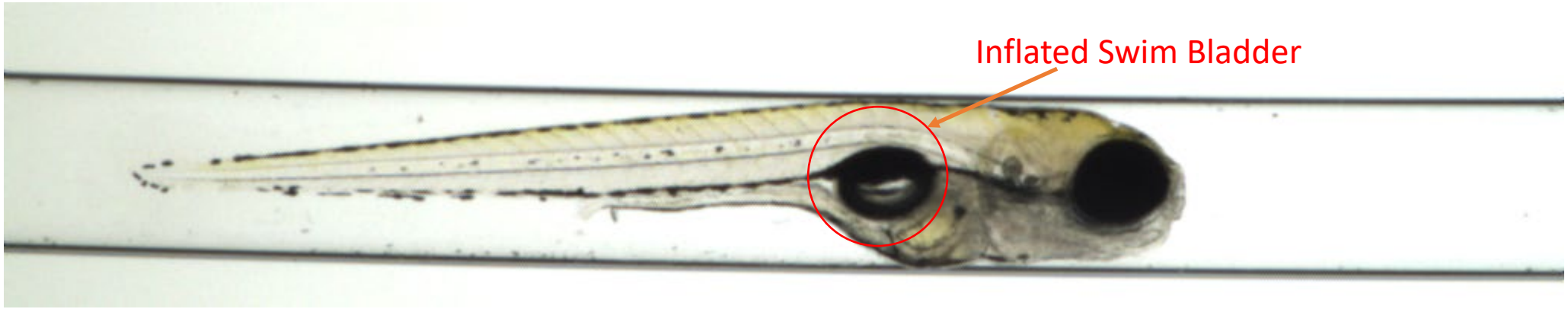


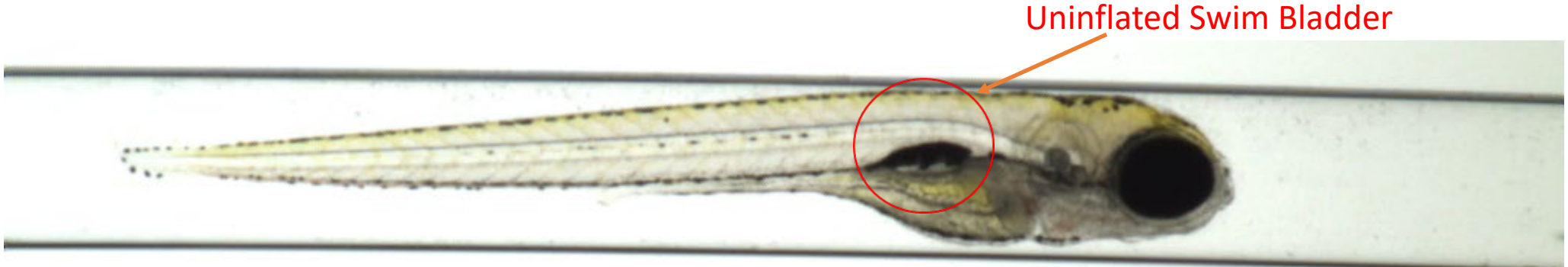
Hill, B.N., Hunter, DL., Jarema, K.A., Britton, K., Hedge, J.M., M Lowery, M., Olin, J., Padilla, S. Poster. Zebrafish Larval Locomotor Activity is Altered by Dimethyl Sulfoxide Under Two Different Exposure Scenarios. Society of Environmental Toxicology and Chemistry (SETAC): North America Annual Meeting. November 15-19, 2020. Virtual.

Swim Bladder

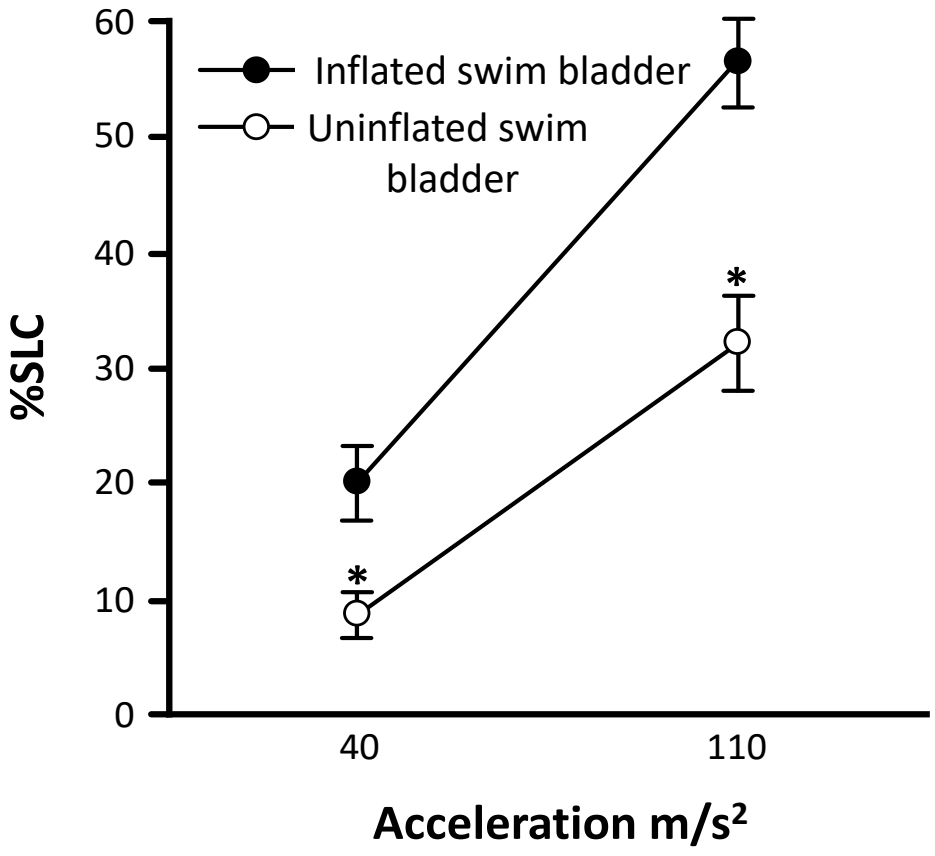
- To inflate the swim bladder, larvae swim to the water surface and gulp air, filling the swim bladder

- Larvae with an inflated swim bladder have the same density as water, allowing them to expend less energy to move and feed

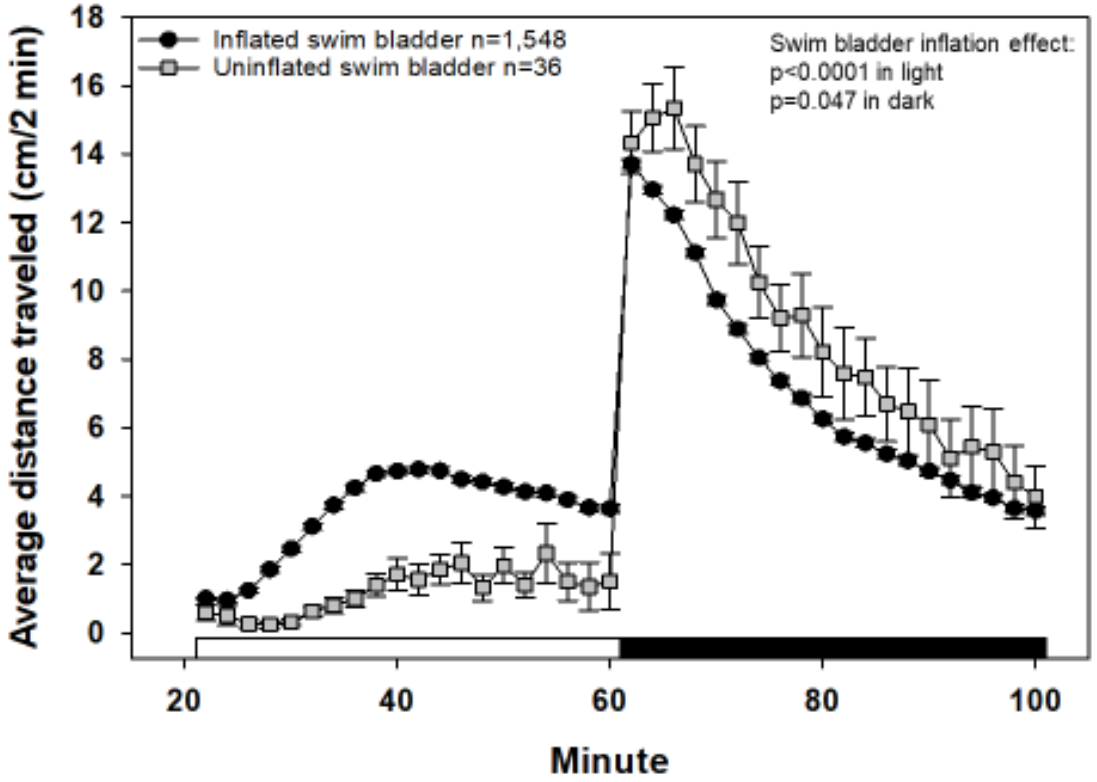




Swim bladder status affects startle response and locomotor activity



Redrawn from Burgess and Granato, 2008



Hill *et al*, 2020, Assessment of Larval Zebrafish Locomotor Activity for Developmental Neurotoxicity Screening, *Experimental Neurotoxicology Methods* (Llorens and Barenys, Eds)

Conclusions about swim bladder inflation

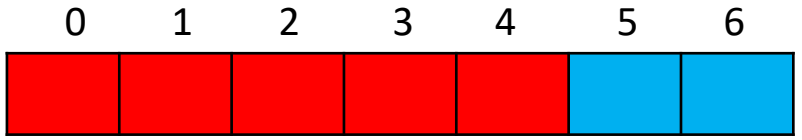
- Profoundly affects locomotor activity and startle response
- Experimental conditions may affect likelihood of swim bladder inflation
  - Amount of solution in the well
  - Changing of plate
  - Shape of the wells (water tension)
- Endpoint for neurotoxicity?
  - Requires autonomic nervous system innervation
  - Requires a behavioral response in the larva

Is this a Test for Developmental Neurotoxicity or Pharmacological Activity of a Chemical?

- Some are screens for Developmental Neurotoxicity
  - Does the chemical affect the development of the nervous system?
    - Function (locomotor activity) is used as an endpoint.
    - Chemical should be present during development, but not during testing
- Some are screens for Neuroactivity (Pharmacological Activity) of a chemical
  - Does the chemical affect the function of the nervous system?
    - Function (locomotor activity) is used as an endpoint
    - Chemical should be present during testing
- Some are screens for both
  - Does the chemical affect either the development or function of the nervous system?
    - Function (locomotor activity) is used as an endpoint.
    - Chemical is present both during development and testing

Chemical Treatment

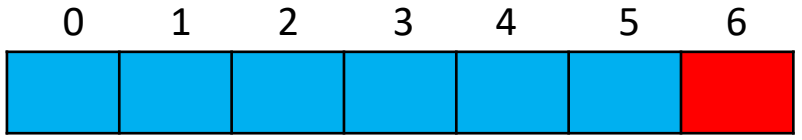
Developmental *Exposure*

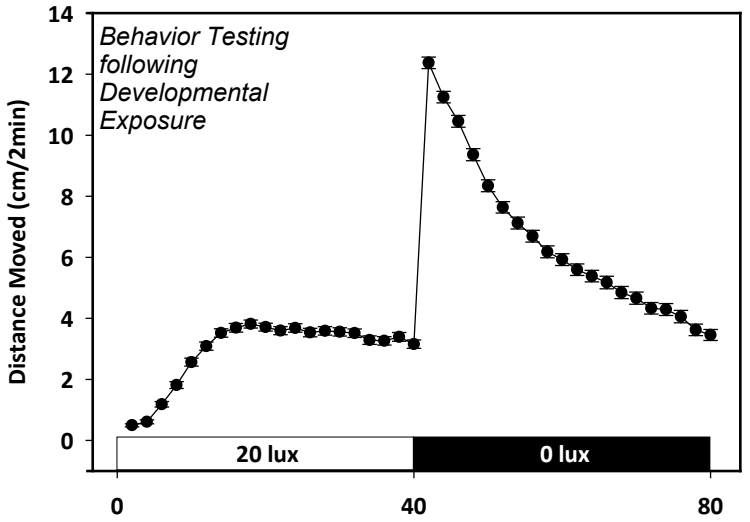


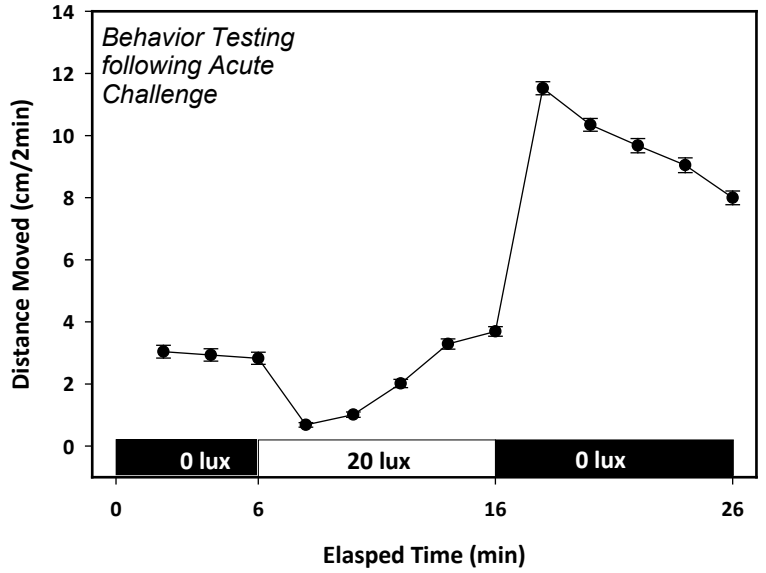
= Chemical Exposure

= No Chemical Exposure

Acute *Exposure*





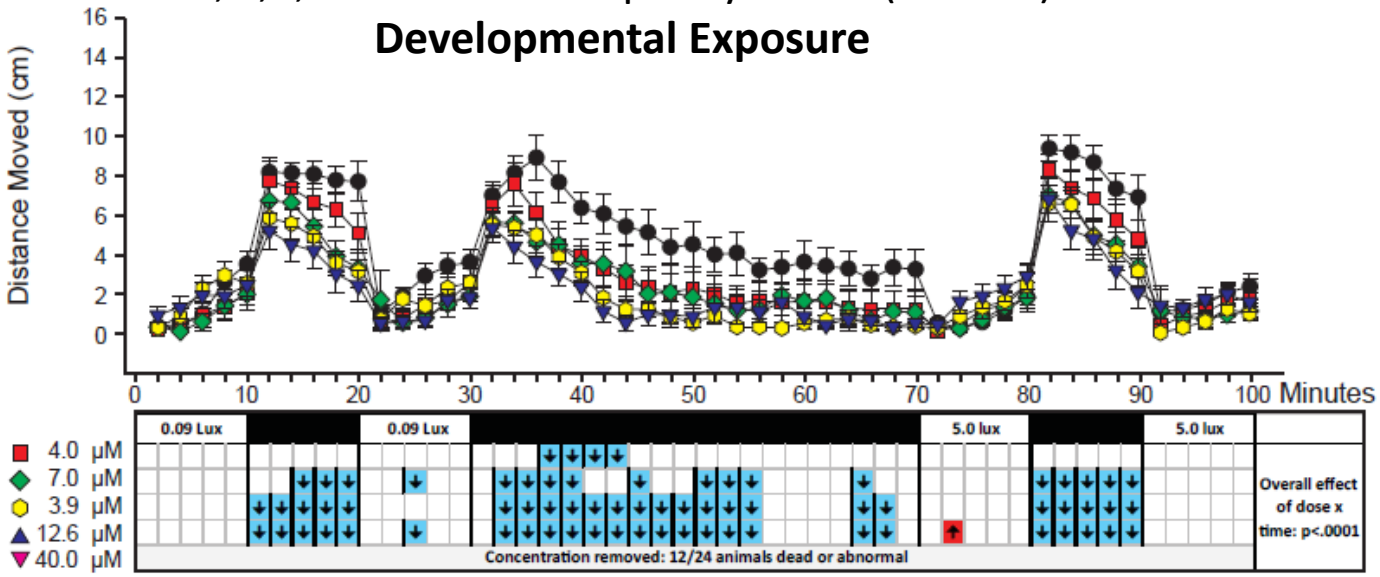


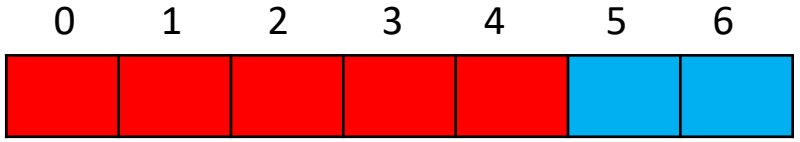
Age | Time | Terata | Light | DMSO | Bladder | **Chemical**

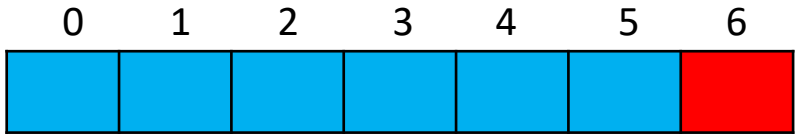
Developmental effects, but minor neuroactive effects

2,2’,4,4’-Tetrabromodiphenyl ether (BDE-47)

Developmental Exposure



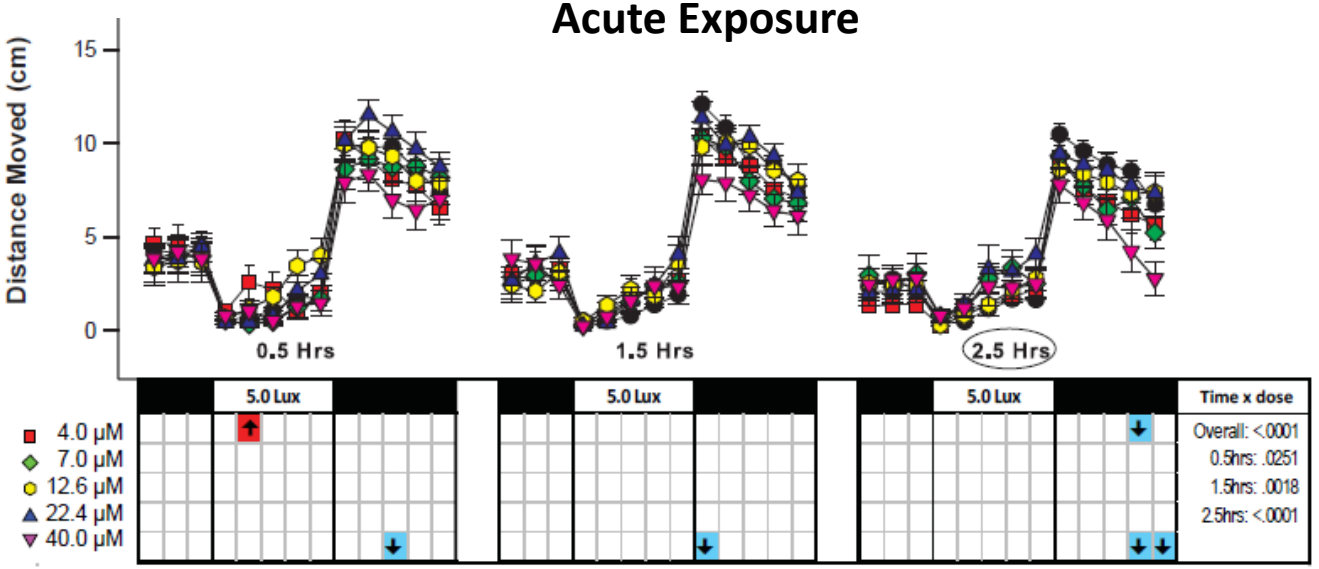




Data from Jarema *et al*, 2015

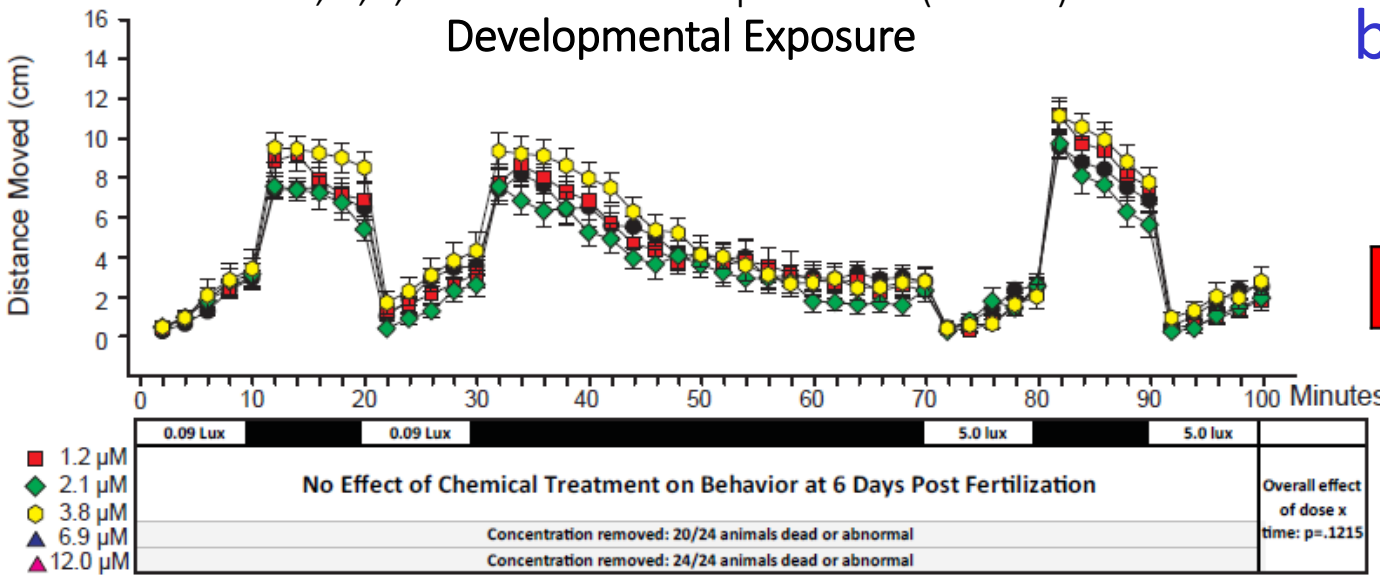
2,2’,4,4’-Tetrabromodiphenyl ether (BDE-47)

Acute Exposure

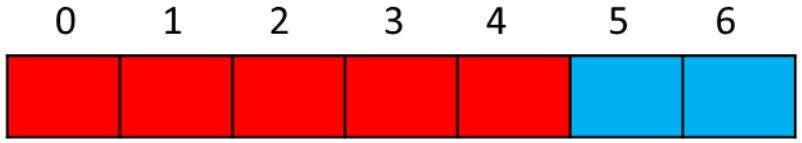


3,3’,5,5’-Tetrabromobisphenol A (TBBPA)

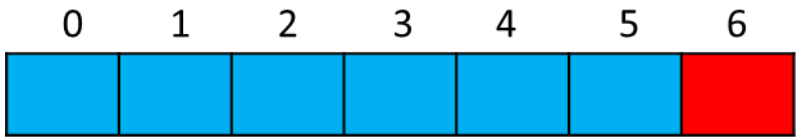
Developmental Exposure



No developmental effects, but marked neuroactive effects

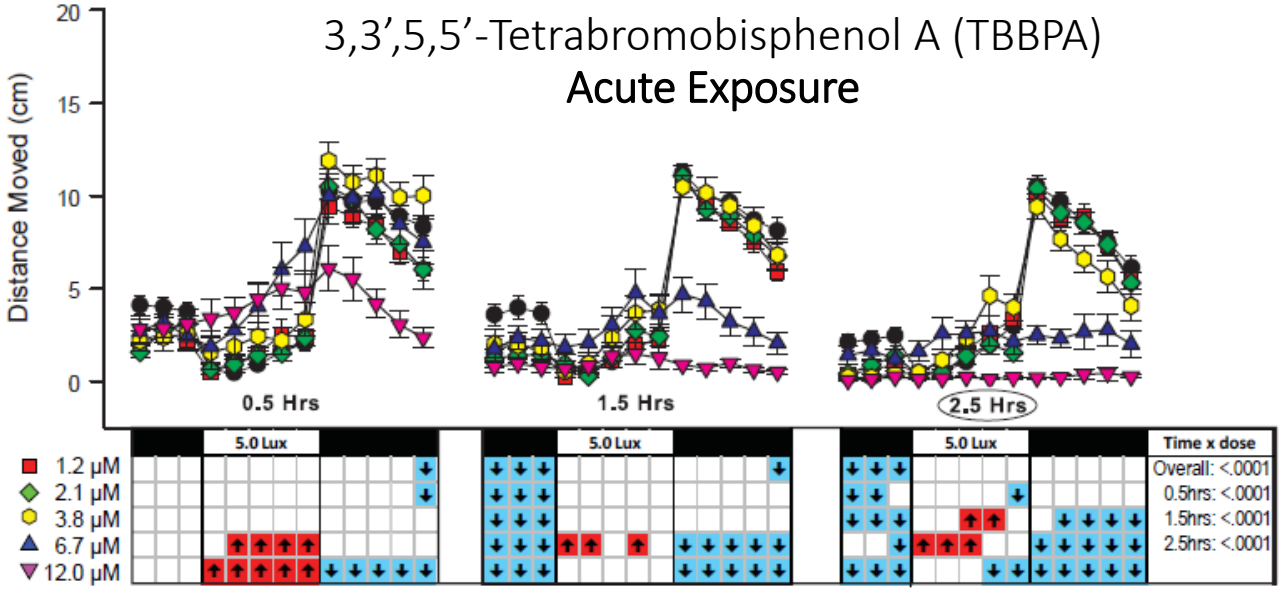


Also shows that rinsing protocol removed chemical



3,3’,5,5’-Tetrabromobisphenol A (TBBPA)

Acute Exposure



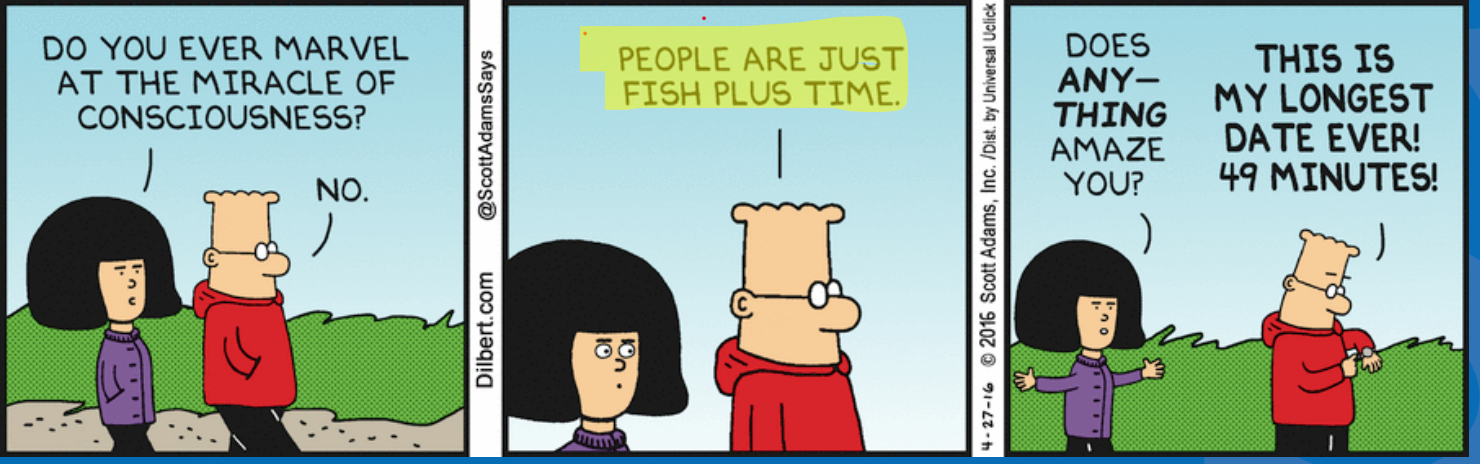
Data from Jarema *et al*, 2015

Summary of Neuroactive vs Neurodevelopmental Considerations

- Larval Behavioral Profile if the chemical is present = developmental effects + neuroactive (pharmacological effects) and it may be difficult to tease the two apart
- Presence of chemical may confound developmental neurotoxicity testing
- Can be experimentally difficult to remove the chemical before testing
- Depends on your scientific question

Summary

- All these variables affect the behavioral profile of larval zebrafish
- May also increase variability of the results
- Recommendations
  - Consistent experimental design
  - Know your assay
    - Which variables make a difference and which do not
  - Consistent reporting of all variables



OECD Working Group on Zebrafish Behavioral Assays

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Ainhoa Alzualde,Arantza Muriana	**Biobide**
Jui-Hua Hsieh, Kristen Ryan, Mamta Behl	**Division of the National Toxicology Program, National Institute of Environmental Health Sciences**
Anna Price	**European Commission -DG Joint Research Centre**
Cindy Woodland	**Health Canada / Government of Canada**
Nils Klüver	**Helmholtz Centre for Environmental Research GmbH - UFZ, Germany**
Lee Ellis	**National Research Council of Canada**
Magdalini Sachana	**Organisation for Economic Co-operation and Development (OECD)**
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Joan Hedge
Terra Irons
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Beth Padnos
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