Application of ToxCast Curve-Fitting Software to Zebrafish Larval Locomotor Response Data

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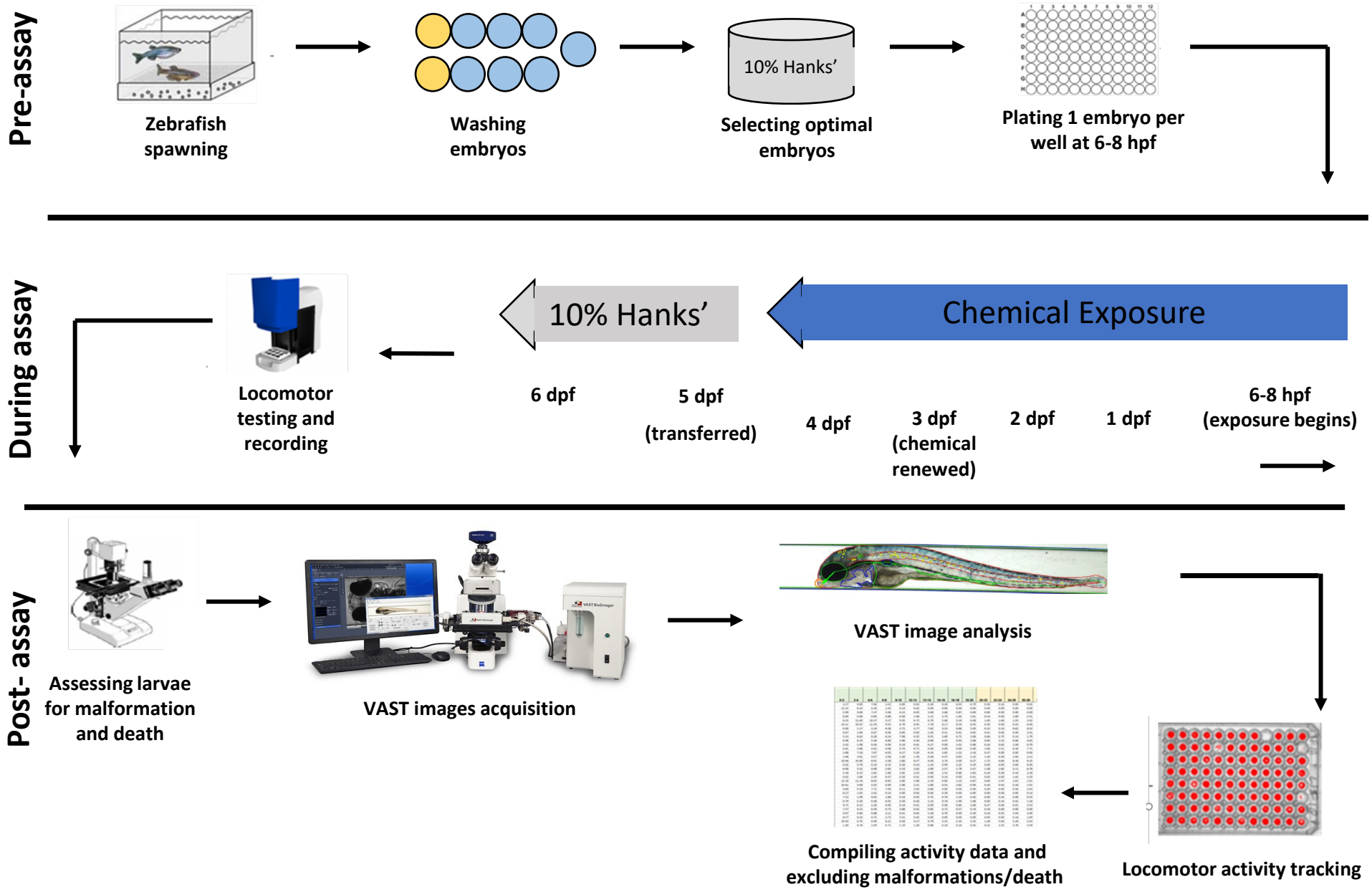
Disclaimer

- The views expressed in this presentation are those of the authors and do not necessarily reflect the views or policies of the U.S. EPA.
- Zachary Rowson was supported by appointment to the Research Participation Program of the U.S. Environmental Protection Agency, Office of Research and Development, administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and the U.S. EPA.

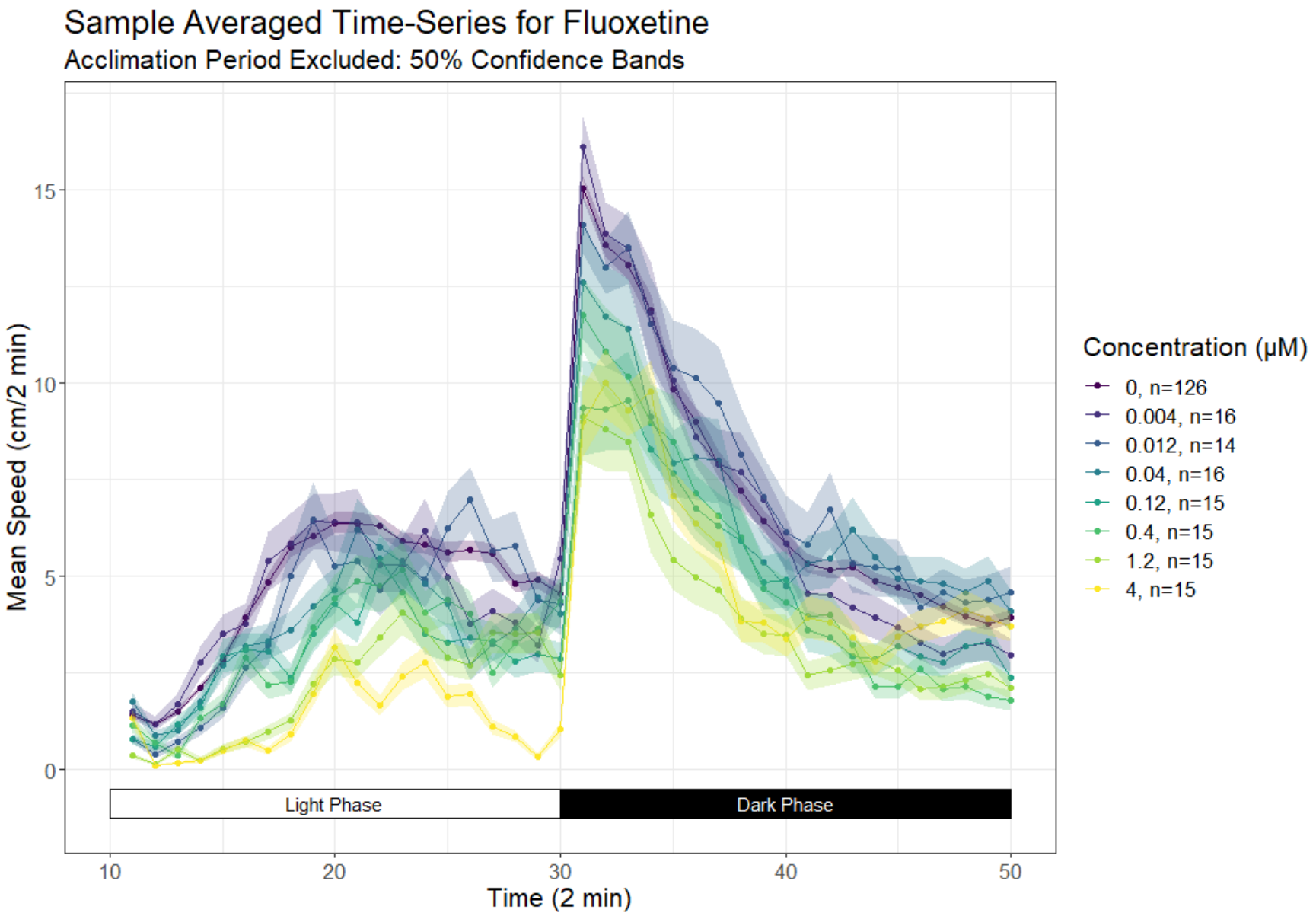
Why Apply ToxCast Curve-Fitting Software to Zebrafish Locomotor Response Data?

- Facilitate integration of DNT NAMs
  - Zebrafish locomotor response (LMR) assay for DNT is a member of the *in vitro* DNT NAM battery at EPA CCTE
  - tcplfit2 is the concentration-response modeling software for bioactivity data in CCTE
  - Pipelining zebrafish behavior data with ToxCast pipeline software would provide zebrafish data analyzed with a standardized software

What is the Zebrafish Locomotor Response Assay?



Example Locomotor Response Data



What is ToxCast's Curve-Fitting Software, tcplfit2?

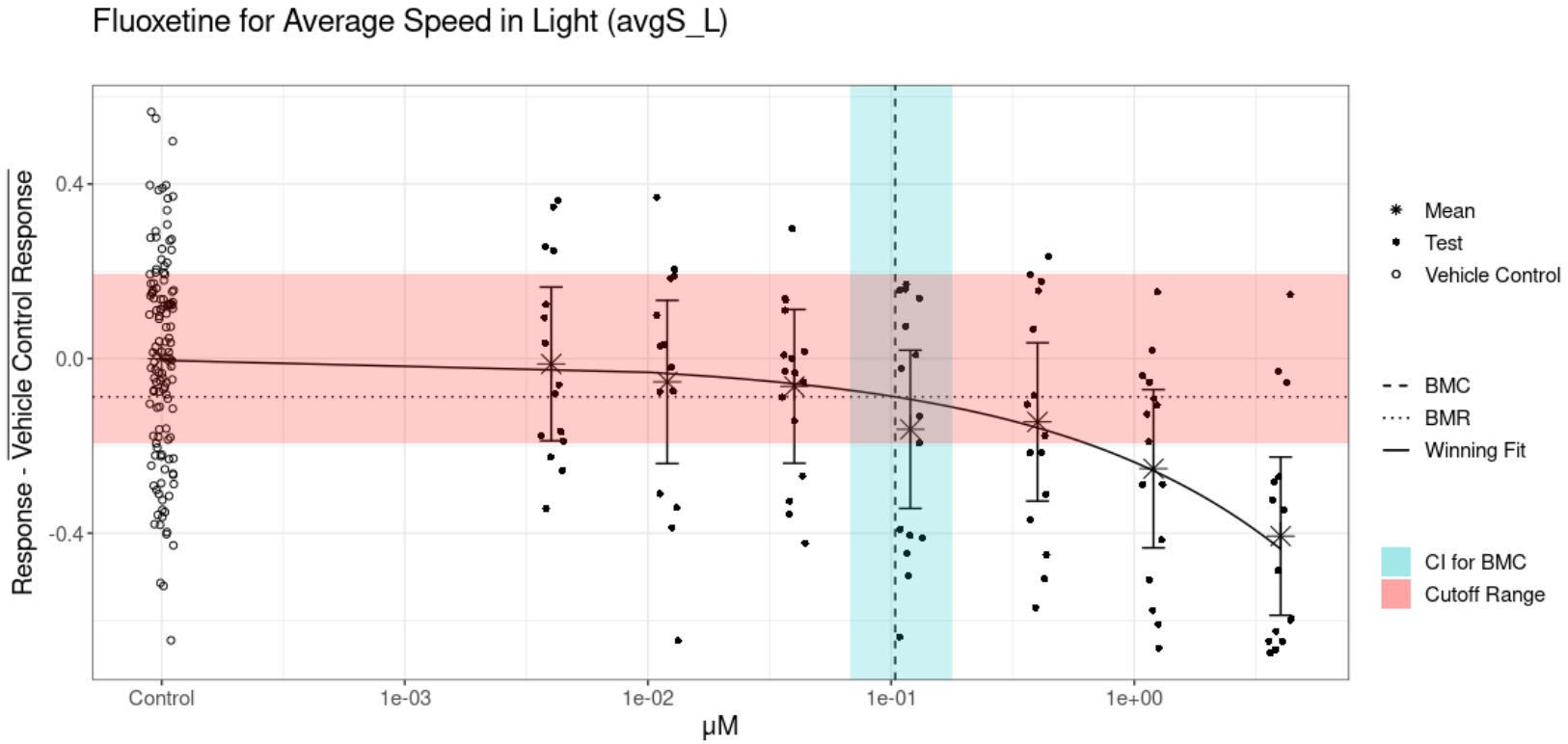
- tcplfit2 is a standalone version of the curve-fitting and hitcalling core of tcpl (ToxCast Pipeline)
- Extended from prior iterations of tcpl's curve-fitting software to include more curve-fitting functions
  - New models are derived from Benchmark Dose modeling software, BMDExpress and BMDExpress 2
  - tcplfit2 includes constant null response model, exponential models with 2 to 5 parameters, 1st and 2nd degree polynomials, power function, hill, and gain-loss function
- Software adds ability to perform benchmark dose modeling to produce continuous potency metrics
- Fits all functions in concentration-response mode to evaluate chemical-effect on assay endpoints
  - Best fitting function is chosen by Aikake Information Criteria (AIC)
- Produces "hitcalls" in continuous interval [0,1] that represent the evidence of chemical activity
  - hitcalls are dependent on the quality of best curve-fit to concentration-response data and the intensity of response in treatment groups

Concentration-Response Models fit by tcplfit2

Model Name	Functional Form	Model Name	Functional Form
cnst	$\mu_i = 0$	poly1	$\mu_i = a * x$
exp2	$\mu_i = a * (e^{\frac{x}{b}} - 1)$	poly2	$\mu_i = a * \left(\frac{x}{b} + \frac{x^2}{b^2}\right)$
exp3	$\mu_i = a * (e^{\left(\frac{x}{b}\right)^p} - 1)$	pow	$\mu_i = a * x^p$
exp4	$\mu_i = tp * \left(1 - 2^{-\frac{x}{ga}}\right)$	hill	$\mu_i = \frac{tp}{1 + (\frac{ga}{x})^p}$
exp5	$\mu_i = tp * \left(1 - 2^{\left(-\frac{x}{ga}\right)^p}\right)$	gnls	$\mu_i = \frac{tp}{(1 + (\frac{ga}{x})^p) * (1 + (\frac{x}{la})^q)}$

Example tcplfit2 Curve-Fit

Example curve-fitting of previously displayed Fluoxetine exposure data for Average Speed in Light endpoint



Output

Model parameters

Best fitting model

cutoff	name	assay	endp	lam.hat	shift	n_gt_cutoff	cutoff.1	fit_method	top_over_cutoff	rmse	a	b	tp	p	q
0.19483	Fluoxetine	CCTE_Padilla_lmrALD	avgS_L	0	1	51	0.19483	pow	2.23975	0.2467024	-0.2371342	NA	NA	0.4399251	NA

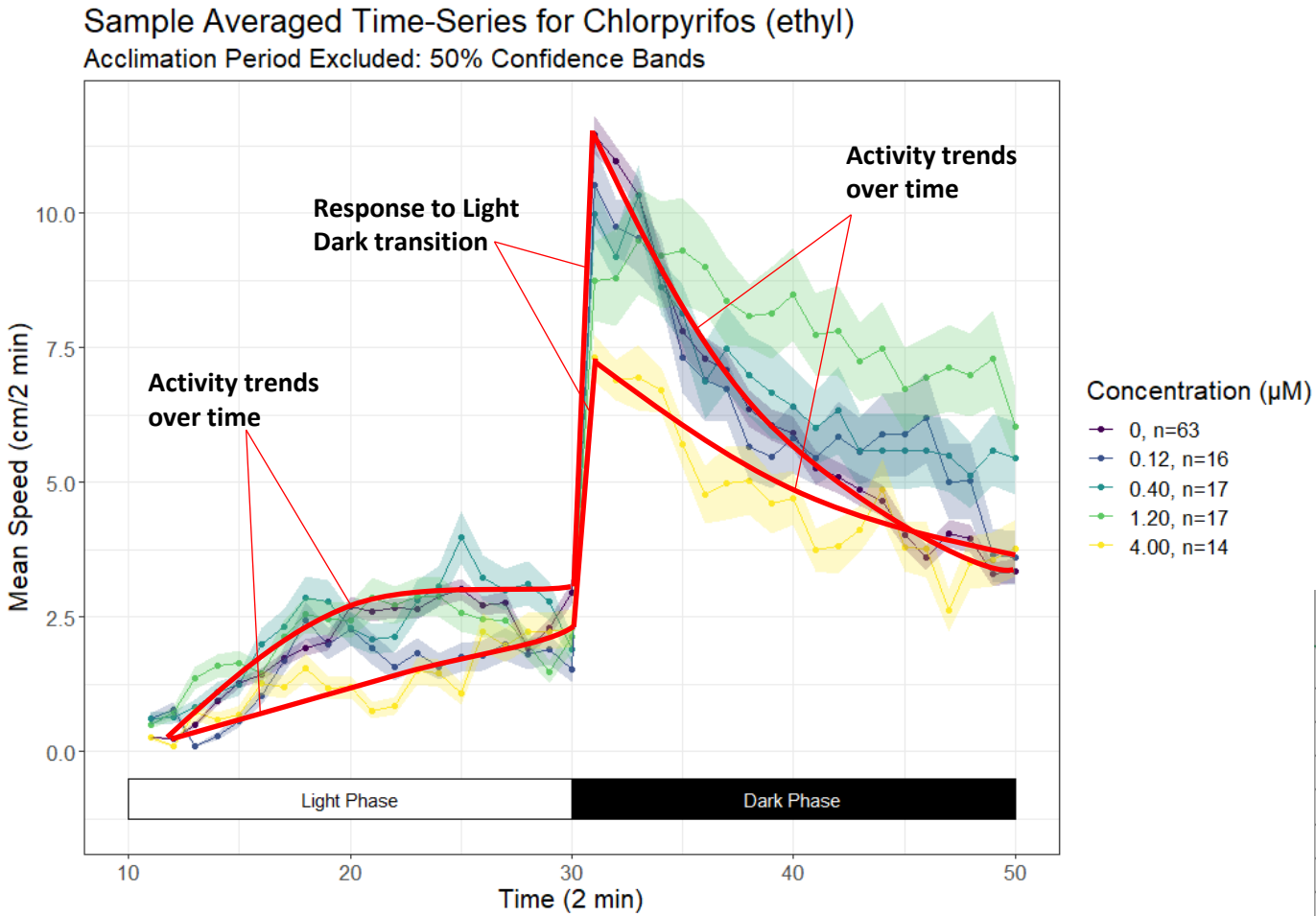
ga	la	er	bmr	bmdl	bmdu	caikwt	mll	hitcall	ac50	ac50_loss	top	ac5	ac10	ac20	acc
NA	NA	-1.525714	0.08760858	0.06743117	0.1797387	3.38338e-10	-8.089382	1	0.8275313	NA	-0.4363707	0.004412352	0.02132778	0.1030911	0.6397587

ac1sd	bmd
0.05265809	0.1039913

Benchmark response and benchmark dose with confidence interval

hitcall

Why is it Difficult to Apply ToxCast Curve-fitting Software to Locomotor Response Data?



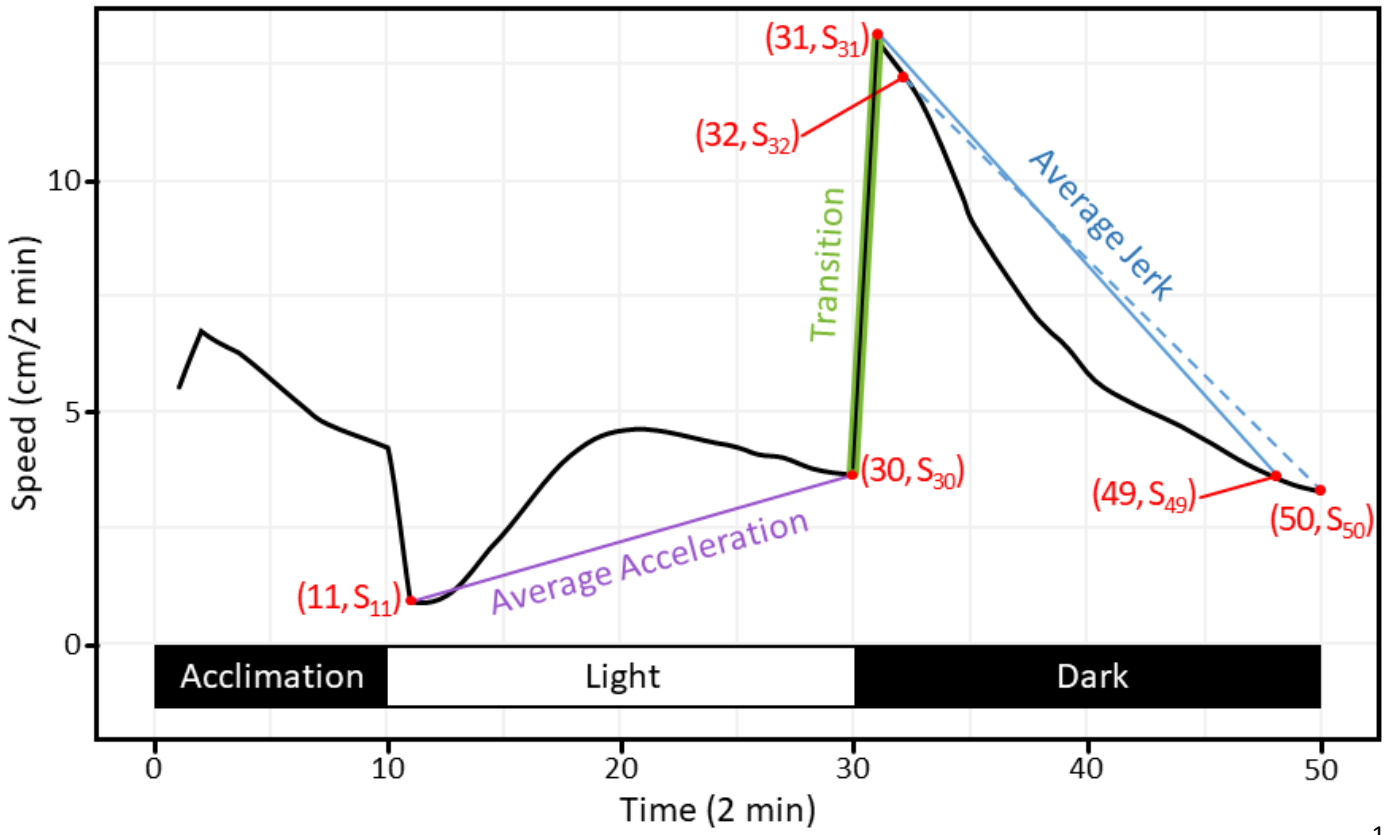
- Curve-fitting of data requires a one-dimensional response
- LMR data exists on a time scale and that time dimension may contain information on changes in activity that occur after chemical exposure
- Typically, only metrics describing net measures of distance traveled by zebrafish are used as a one-dimensional response
  - Example endpoints: Average Speed and Total Distance Moved
- However, summation over time removes characteristics of LMR data that exist on time scale
  - Such as zebrafish activity trends and zebrafish response to Light-Dark transition

Chemical (µM)	N	*Light Total*	*Dark Total*
Chlorpyrifos 79-84	**63**	**0.1197**	**0.1074**
0.12	16	0.2089	0.5914
0.4	17	0.6592	0.6634
1.2	17	0.1368	0.0502
4	14	0.0476	0.1696
12	2	*N=2 Dose removed from stats and graphs.*	
40	0	*N=0 Dose removed from stats and graphs.*	
120	0	*N=0 Dose removed from stats and graphs.*	

Above: Non-parametric statistical test of total movement per illumination phase does not indicate Chlorpyrifos exposure perturbates zebrafish behavior

Endpoints Evaluated

(i, S_i); $i = i^{th}$ time interval, S_i = speed at i^{th} time interval



Startle vs Average Speed in Light

$= strtlAavg = s_{31} - avgS_L$

Startle Fold-Change

$= strtlF = \frac{s_{31}}{s_{30}}$

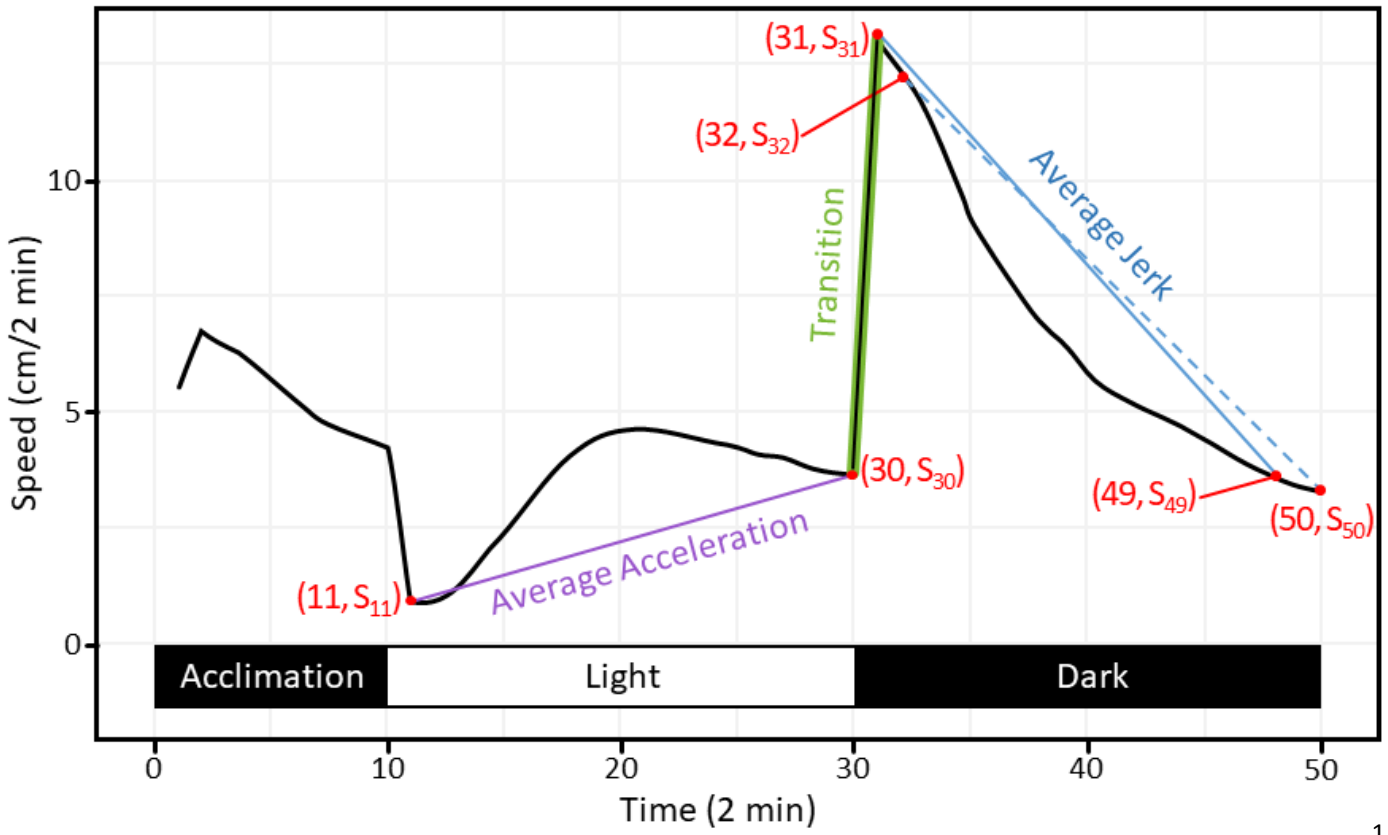
Habituation in Dark[1]

$= hbt\_D = \frac{s_{50}}{s_{31}}$

- Solution: Identify endpoints that describe highly reproducible characteristics of the zebrafish locomotor response behavior potentially lost during dimension reduction
  - Evaluate if these endpoints can be perturbed by chemical exposure
- Endpoints were either developed for this work or observed in literature
- Endpoints describe zebrafish's
  - Reaction to Light-Dark transition (startle)
  - Acclimation to new light conditions (habituation)

Derivation of Endpoints

(i, S_i); i = i^{th} time interval, S_i = speed at i^{th} time interval



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$= strtlAavg = s_{31} - avgS_L$

Startle Fold-Change

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Habituation in Dark[1]

$= hbt\_D = \frac{s_{50}}{s_{31}}$

- Average Acceleration is the average change in speed from one time interval to the next
  - Visually displayed to left as the slope of purple line
- Average Jerk is the average change in acceleration from one time interval to the next
  - Visually displayed to left as the difference in slopes of the two blue lines
- 3 endpoints were constructed to describe startle behavior
  - Startle acceleration is the slope of the green line
  - Other startle endpoints are shown formulaically below figure to left
- "Habituation" endpoint describes change in zebrafish activity over time
  - Wong K et al. 2010
- Most endpoints are calculated for Light and Dark phase separately

Application of tcplfit2 to Endpoint Data

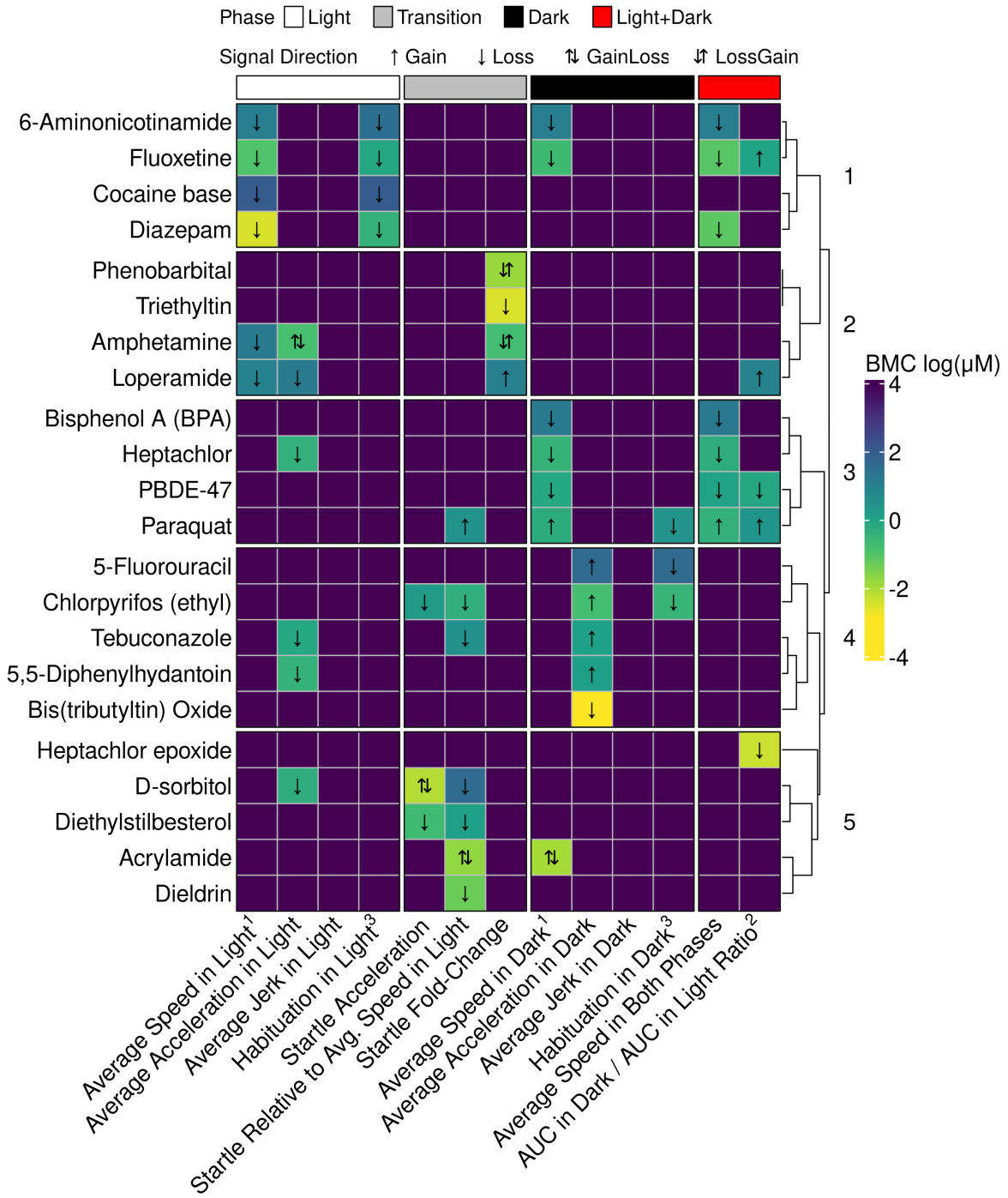
- tcplfit2 requires a measure of background variability in the data to distinguish between noise and signal
  - Endpoint data was symmetrized about mean values prior to evaluation of background variability to allow for bidirectional curve-fitting
  - Measure of background variation (cutoff) is defined as

    3*SE(vehicle control response)
- Benchmark response is defined as 1.349*SE(vehicle control response)
  - 1.349*SE(vehicle control response) is associated with a 10% change in mean response
    - Sheffield et al. 2021

Chemical Set Evaluated

- This workflow was applied to data from multiple concentration exposures of zebrafish to 61 chemicals
  - Set of chemicals was adapted from screening of a 67-chemical evaluation set in high content imaging and microplate reader assays assessing key neurodevelopmental processes
    - Harrill et al. 2018
- 48 of 61 chemicals have potential evidence of *in vivo* DNT in mammals
  - Chemicals were identified in literature review of peer-reviewed *in vivo* mammalian studies for developmental neurotoxicity
    - Mundy et al. 2015
- 13 of 61 chemicals were identified as putative DNT negative controls
  - Chosen due to absence of effect in USEPA ToxCast *in vitro* bioactivity assays

    or
  - Chosen due to lack of evidence of developmental neurotoxicity in a review of the published literature
  - Identification of these chemicals described in more detail in Harrill et al. 2018
  - Negativity of these chemicals in NAM assays for DNT is in question
    - Manuscript in progress detailing efforts to identify list of “negative” chemicals for an evaluation set for DNT NAM accuracy
      - Martin et al. (unpublished)

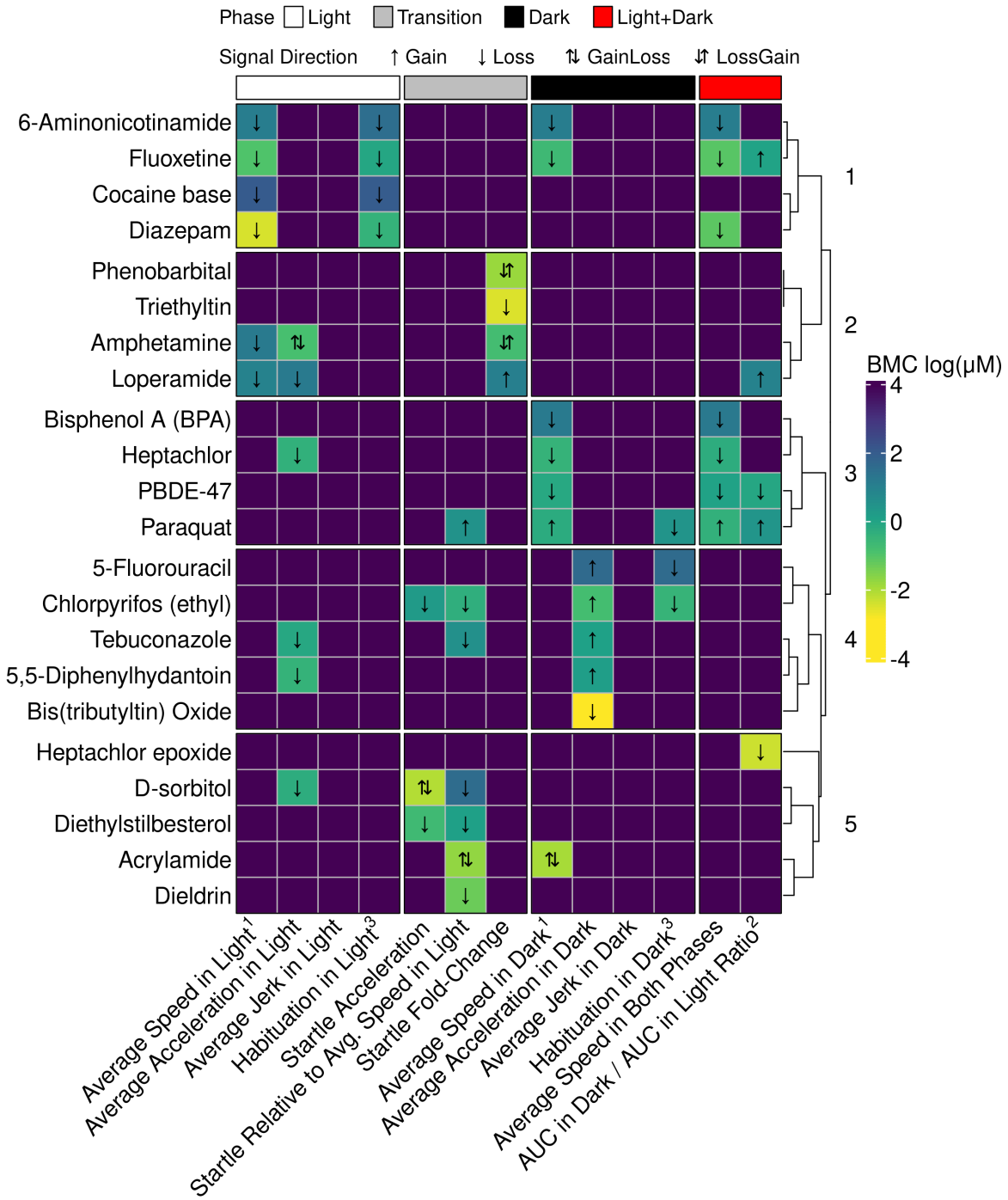


Results

- Left, BMCs produced by application of workflow to 61 chemical set
- **Note:** Only chemicals with hitcall > 0.8 in at least one endpoint are shown
- Columns of Heatmap indicate assay endpoints
- Endpoints are ordered by assay illumination phase, illumination phase is indicated by colored annotation bar at top
  - Combination of Light and Dark phase data is called Light+Dark
- Most added endpoints were perturbed by chemical exposure
  - Average Jerk was only endpoint where chemical activity was not detected
  - No one endpoint was significantly more sensitive than others
- 39/61 (64%) of tested chemicals were found inactive
  - Names of these chemicals are shown below

Chemicals Inactive in Locomotor Response Assay for DNT

6-Propyl-2-thiouracil	Glyphosate
Acetaminophen	Haloperidol
Aldicarb	Hexachlorophene
Amoxicillin	Hydoxyurea
Arsenic	Isoniazid
Cadmium chloride	Lead acetate
Caffeine	Maneb
Captopril	Manganese
Carbamazepine	Methotrexate
Chloramben	Naloxon
Chlorpyrifos (ethyl) oxon	Nicotine
Colchicine	Permethrin
Cotinine	Phenol
Cyclophosphamide	Saccharin
Cytosine arabinoside	Sodium benzoate
Deltamethrin	Sodium fluoride
Dexamethazone	Terbutaline
Di(2-ethylhexyl)phthalate (DEHP)	Thalidomide
Diethylene Glycol	Valproate
Fluconazole	



Results

- Clustering of chemicals by potency metrics appears to reveal patterns in the data.
  - **Cluster 1** chemicals predominantly affect the Light, Dark, and Light+Dark phases.
  - **Cluster 2** chemicals predominantly affect the Light phase and Transition phase.
  - **Cluster 3** chemicals predominantly affect the Dark and Light+Dark phases.
  - **Cluster 4** chemicals affect rate of change metrics, most notably Average Acceleration in Dark.
  - **Cluster 5** chemicals predominantly affect the transition phase with one outlier, Heptachlor Epoxide.
  - **Cluster 6** is not shown and contains chemicals that were not active in the Zebrafish locomotor response assay

- Application of the analysis procedure to a set of reference chemicals could elucidate activity profiles associated with known modes of action or neurological diseases.

Evaluation of Assay Accuracy

- Evaluation of endpoint set improves assay accuracy relative to evaluation set of potential mammalian *in vivo* positives and putative DNT negatives
  - Accuracy improves from 36% to 51%
  - Improvement in accuracy is largely due to change in assay sensitivity from 21% to 42%
  - Assay specificity changes from 92% to 85%, false positives: Loperamide and D-Sorbitol
    - Evaluation of added endpoint set results in one additional false positive, D-Sorbitol

Confusion Matrix Using Total Distance Endpoints Only

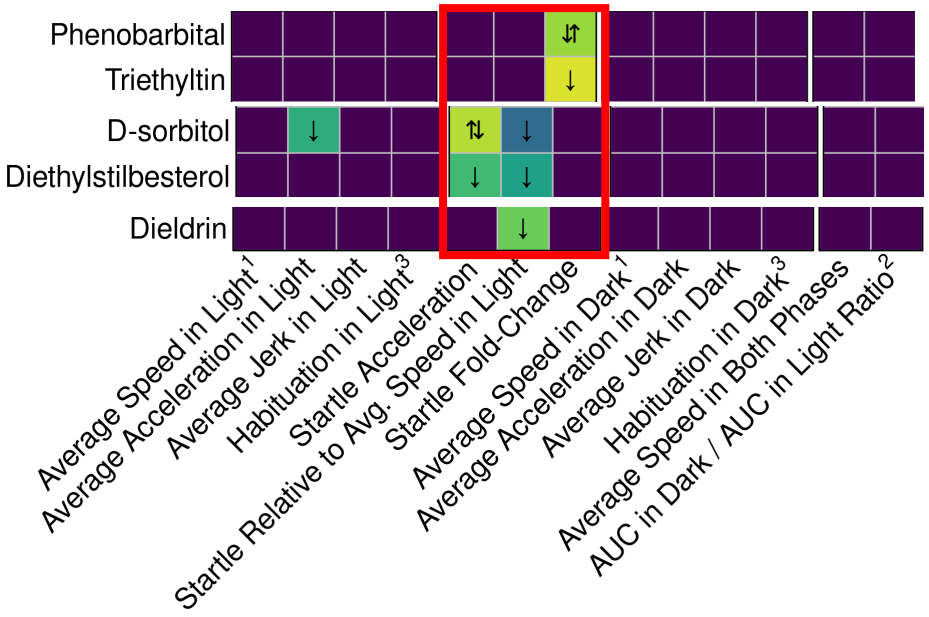
	In vivo DNT positive	Putative DNT negative
Positive	**10**	**1**
Negative	**38**	**12**

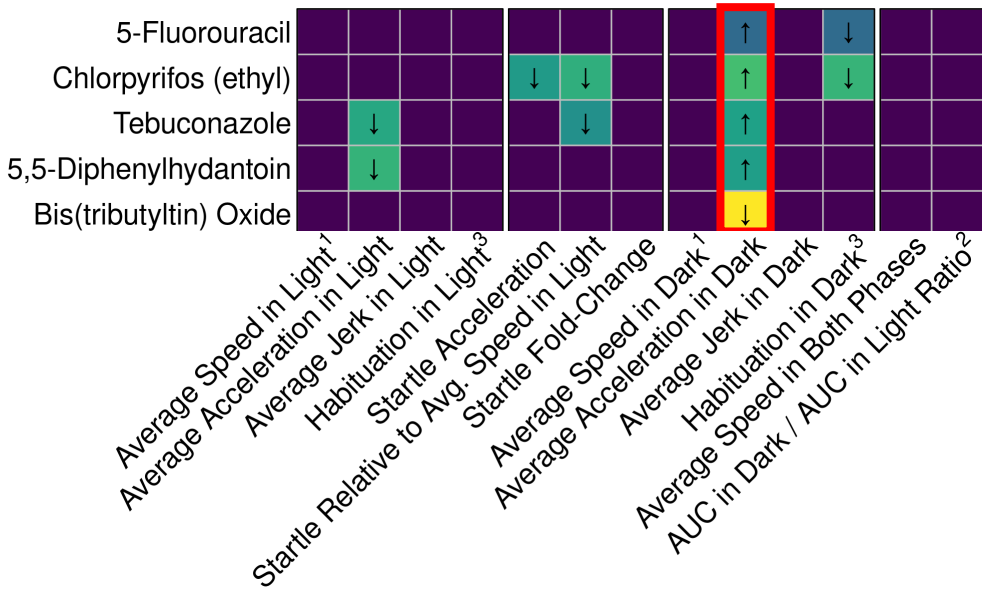
Confusion Matrix Using All Endpoints Tested

	In vivo DNT positive	Putative DNT negative
Positive	**20**	**2**
Negative	**28**	**11**

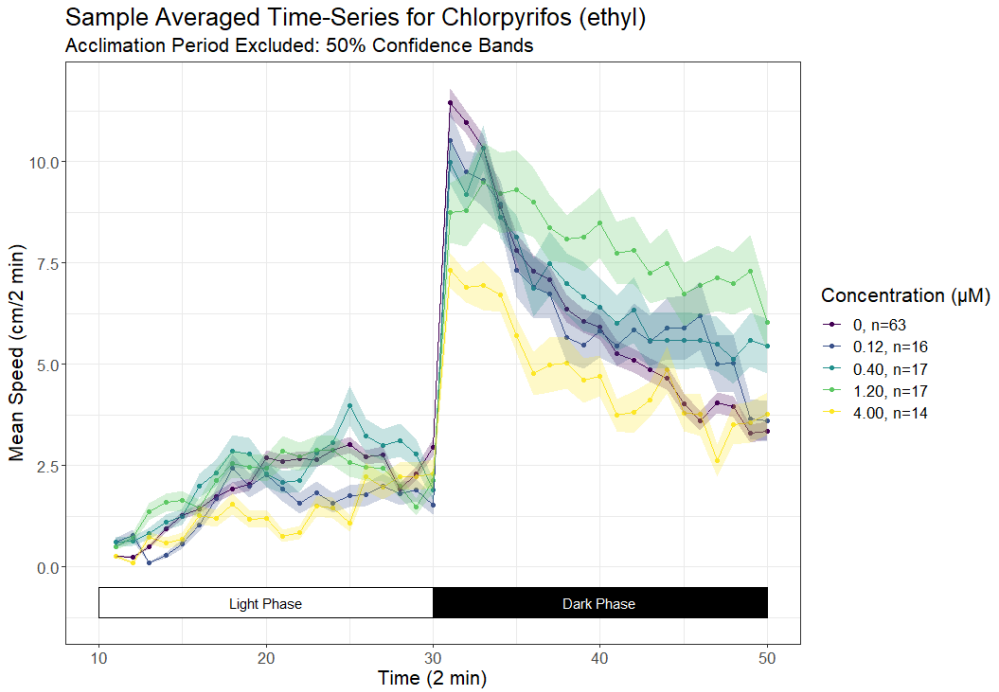
Where is Additional Activity Being Detected?

- Additional activity is primarily being detected in endpoints that evaluate zebrafish startle at the Light-Dark transition and in endpoints that describe changes in activity trends in the Dark phase
- 11 additional chemicals were detected as positive
  - Of these 11, 10 were active in endpoints evaluating the startle response
  - 5 active in Average Acceleration in Dark endpoint that evaluates zebrafish habituation after startle



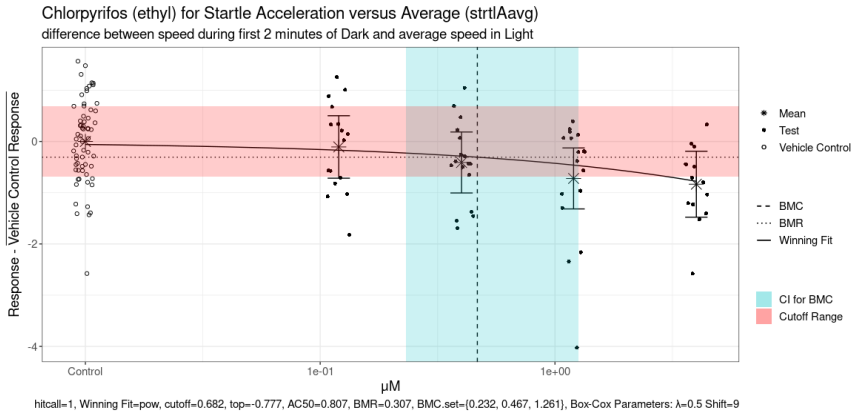


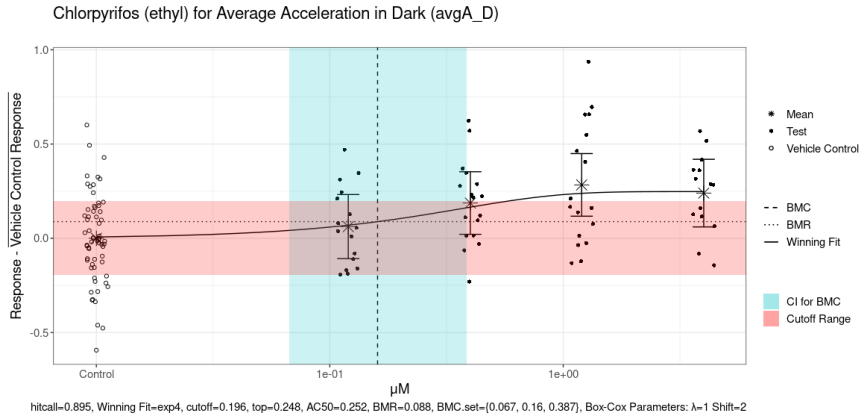
Additional Chemicals Declared Active



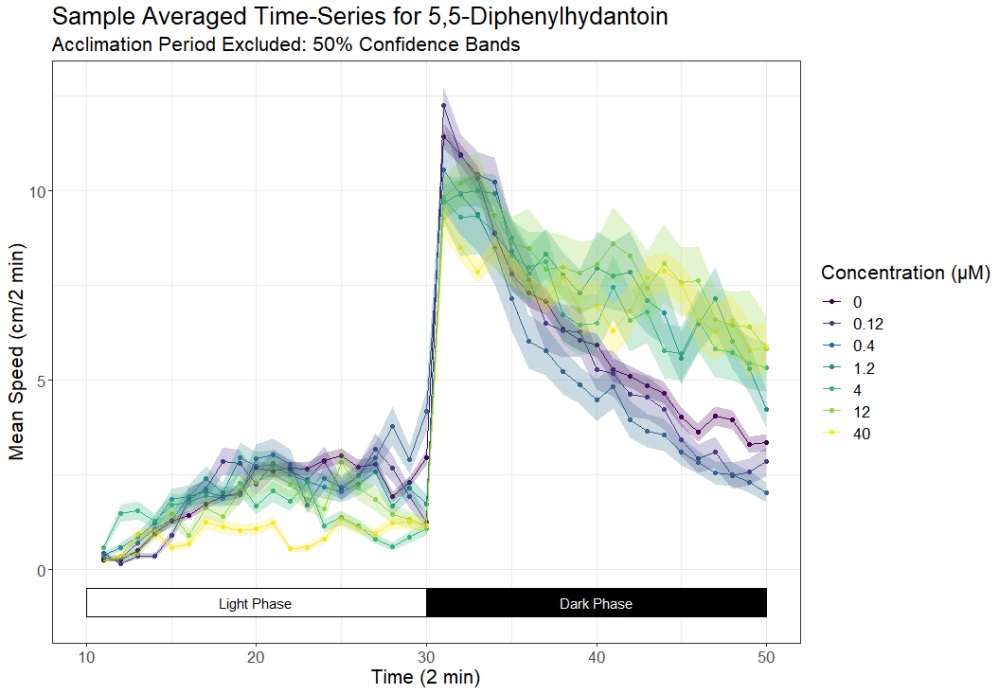
Chlorpyrifos (ethyl)

- Often considered active in zebrafish LMR assay for DNT
- Not detected as active in endpoints describing net activity
- Added endpoints detect changes in zebrafish activity trends with time in Dark and zebrafish startle response



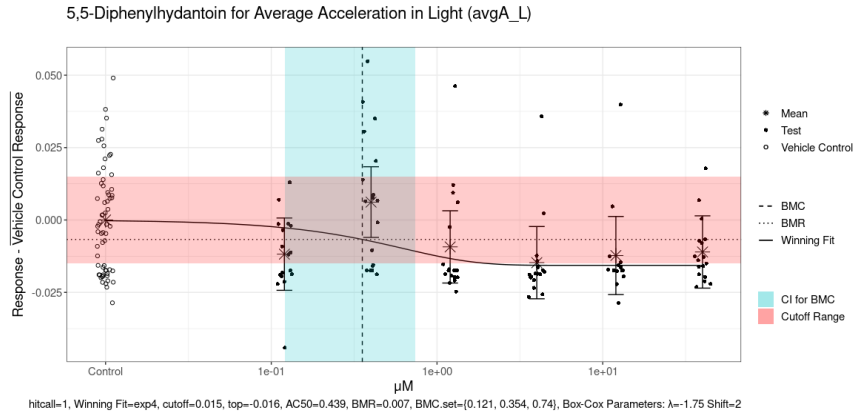


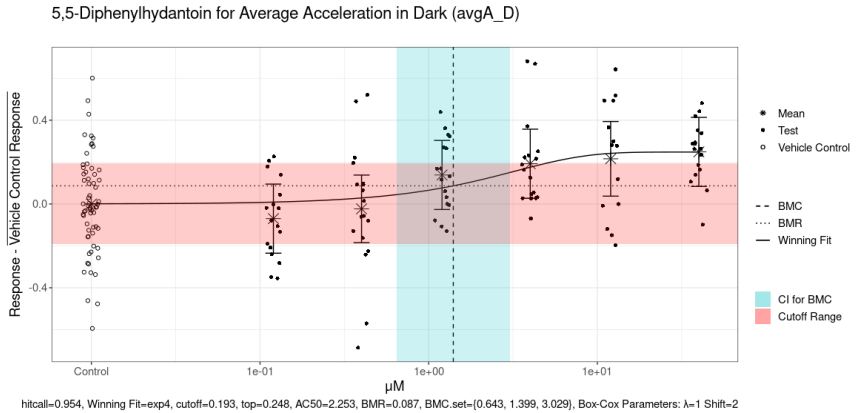
Additional Chemicals Declared Active



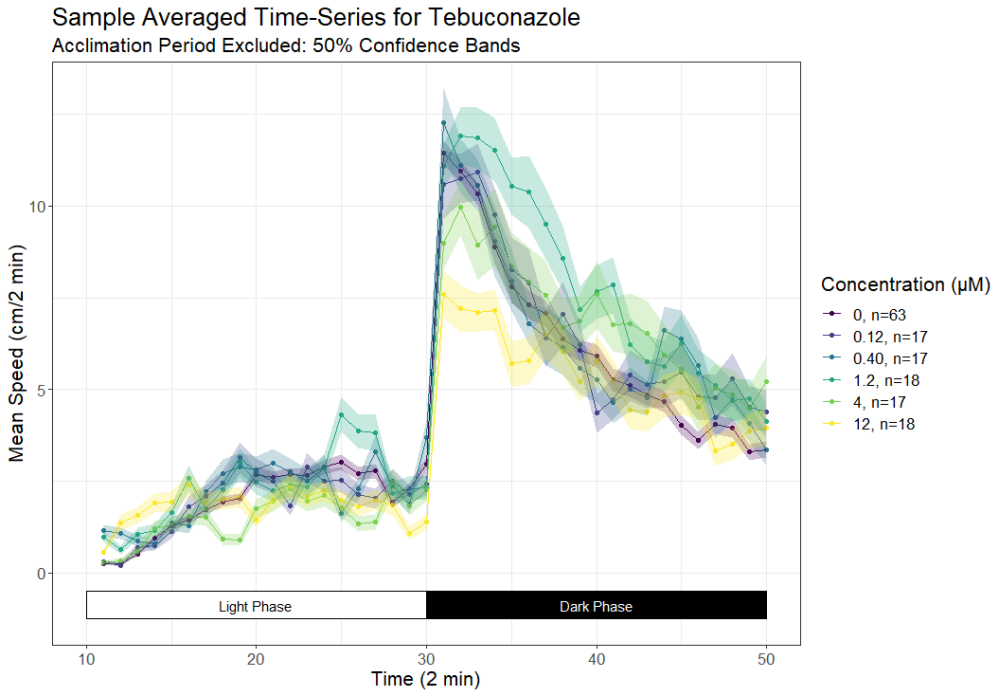
5,5-Diphenylhydantoin

- Not detected as active in endpoints describing net activity (Average Speed)
- Added endpoints detect changes in zebrafish activity trends with time in Light and Dark



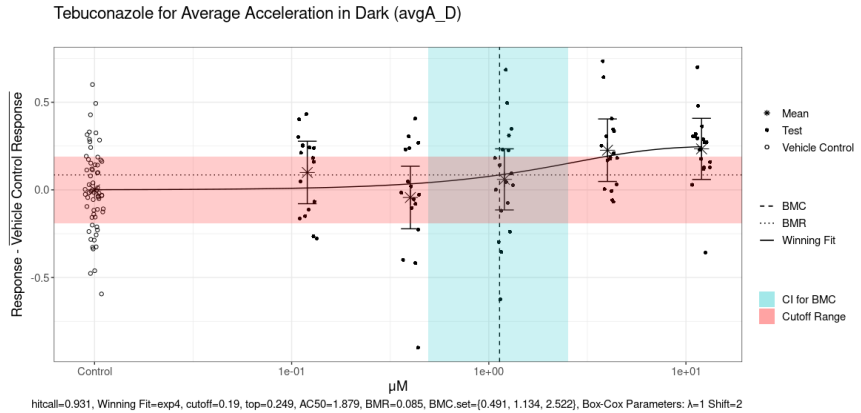


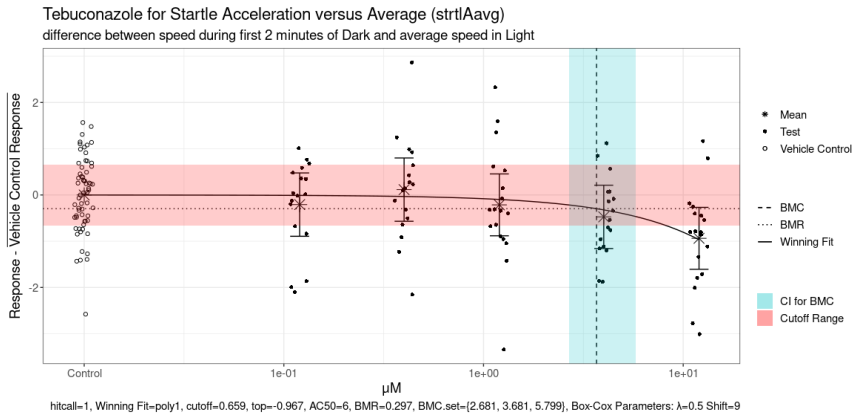
Additional Chemicals Declared Active



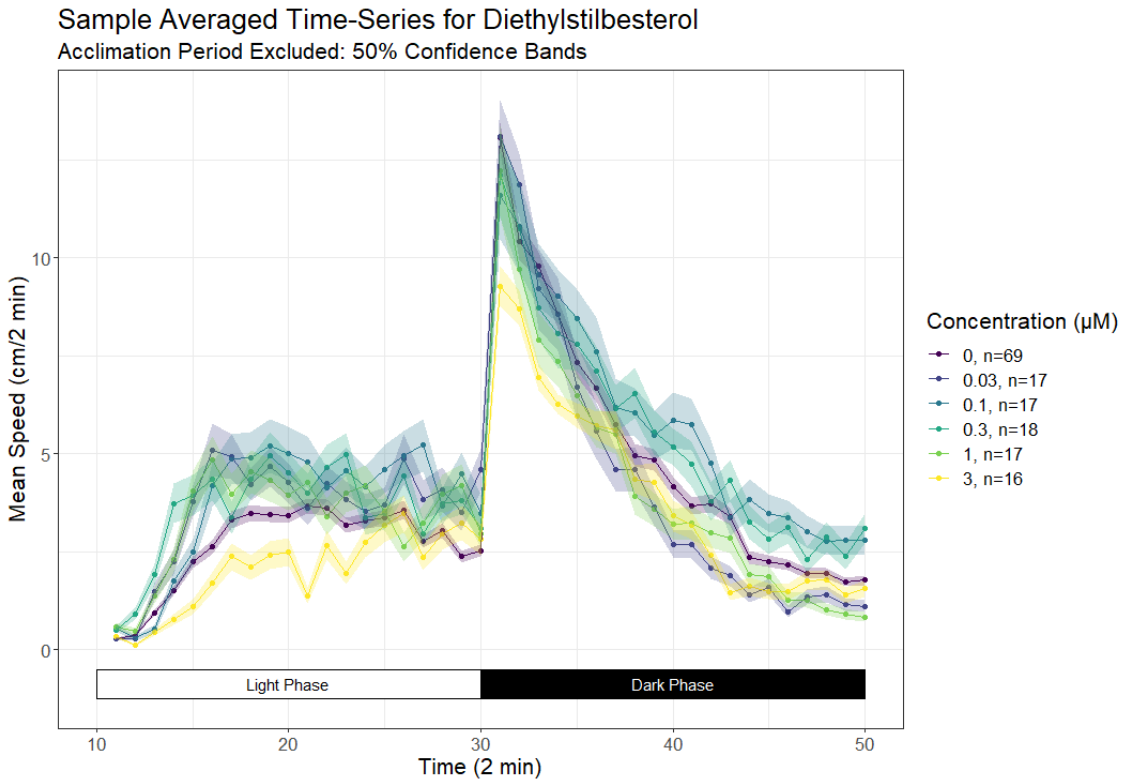
Tebuconazole

- Not detected as active in endpoints describing net activity (Average Speed)
- Added endpoints detect changes in zebrafish activity trends with time in Dark and zebrafish startle response



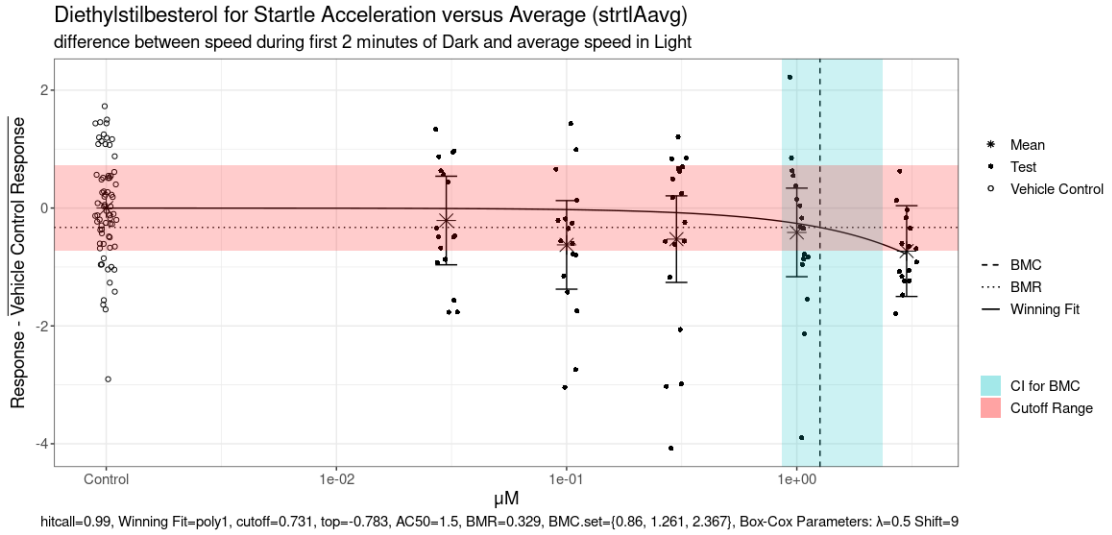


Additional Chemicals Declared Active

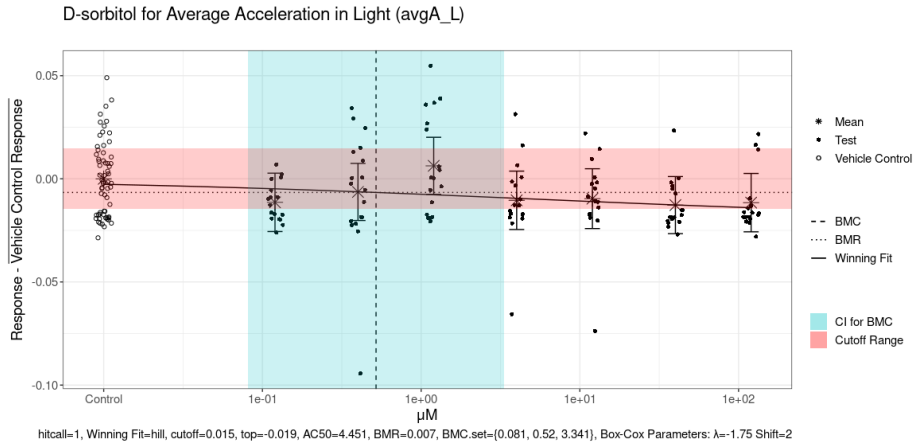


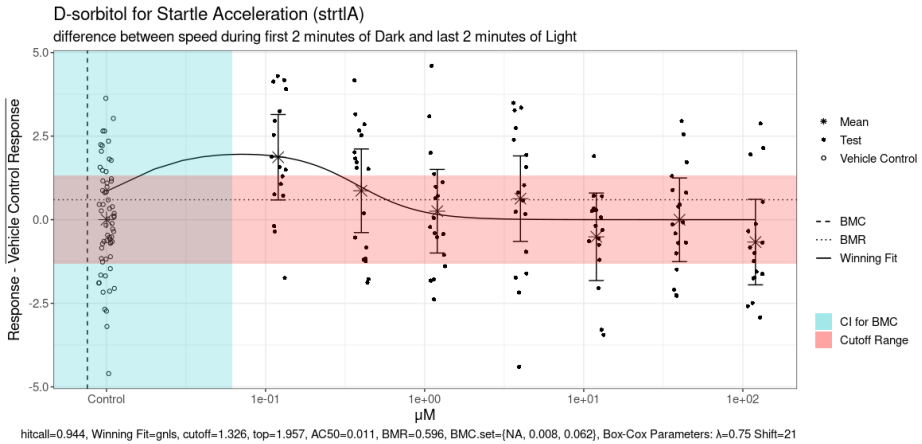
Diethylstilbesterol

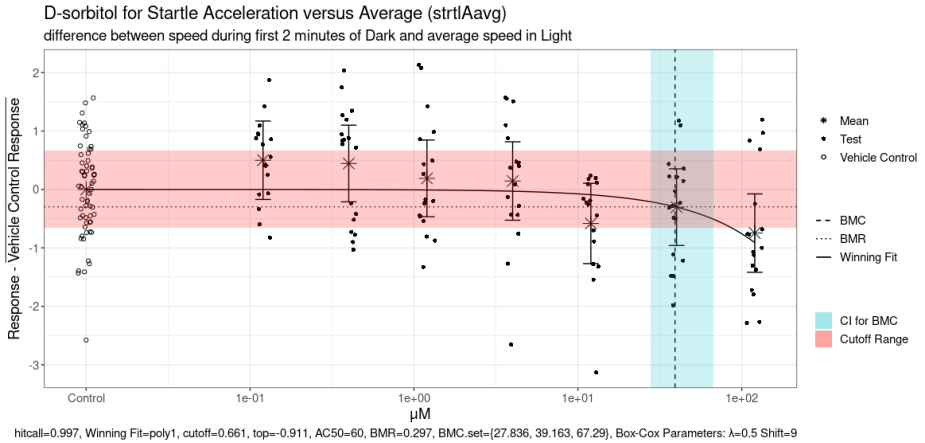
- Not detected as active in endpoints describing net activity (Average Speed)
- Added endpoints detect changes in zebrafish startle response



Assessment of False Positives



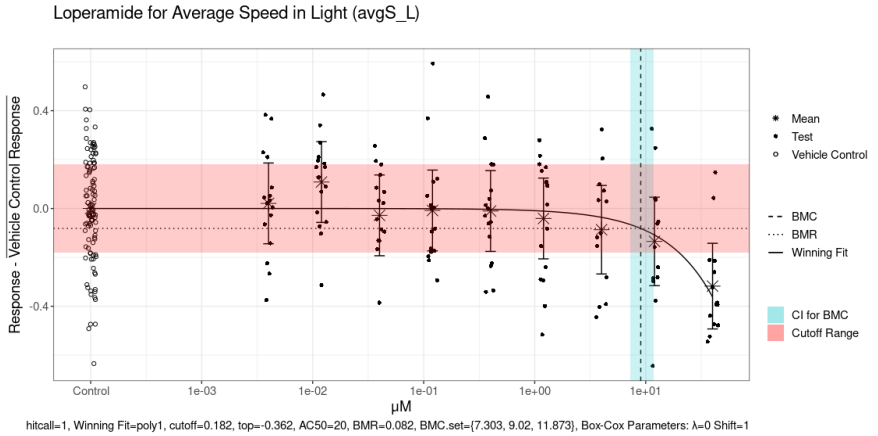




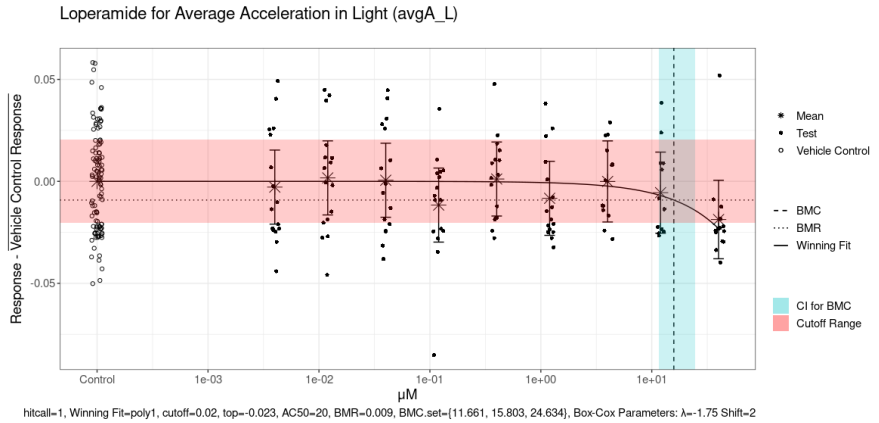
D-Sorbitol

- Evidence of toxicity to neurons
  - Martin at al. (unpublished)
- BMCs associated with active endpoints cover a wide range of concentrations, some BMCs have large confidence intervals
  - Average Acceleration in Light: **BMC** = 0.5 µM CI: 0.08 µM to 3 µM
  - Startle Acceleration: **BMC** = 0.008 µM CI: NA to 0.062 µM
  - Startle Acceleration vs. Average Speed in Light: **BMC** = 28 µM CI: 39 µM to 67 µM
- Variability of BMCs across endpoints and accuracy around BMC estimates may be useful for assessing confidence of chemical activity

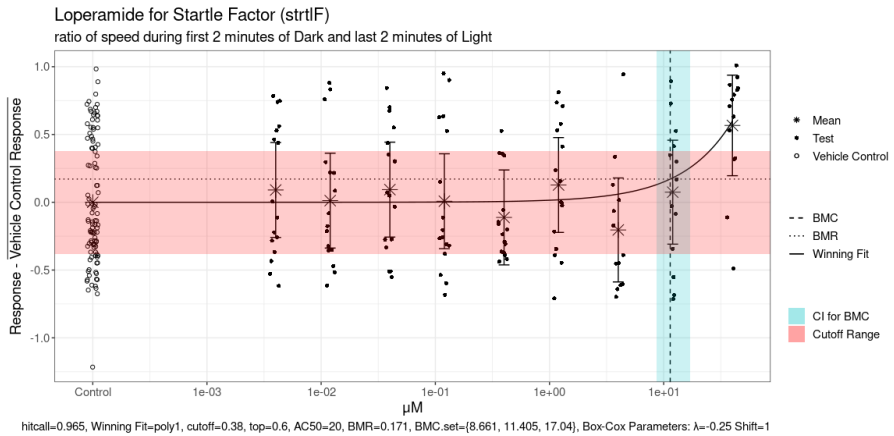
Assessment of False Positives



hitcall=1, Winning Fit=poly1, cutoff=0.182, top=-0.362, AC50=20, BMR=0.082, BMC.set={7.303, 9.02, 11.873}, Box-Cox Parameters: λ=0 Shift=1



hitcall=1, Winning Fit=poly1, cutoff=0.02, top=-0.023, AC50=20, BMR=0.009, BMC.set={11.661, 15.803, 24.634}, Box-Cox Parameters: λ=-1.75 Shift=2



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Loperamide

- Believed to elicit response in neurons, difficulty penetrating blood-brain barrier
  - Martin at al. (unpublished)
- Loperamide used as a positive control in some DNT NAMs
- Evidence of chemical effect on behavior at high concentration and evidence of concentration response behavior in endpoints
  - Average Speed in Light: **BMC** = 9 µM CI: 7 µM to 12 µM
  - Average Acceleration in Light: **BMC** = 16 µM CI: 12 µM to 25 µM
  - Startle Factor: **BMC** = 9 µM CI: 11 µM to 17 µM
- BMCs produced by three active endpoints fall within a close range
- Developmental paradigm could be exposing zebrafish to chemical before blood-brain barrier is fully operational

Are Some Hits Spurious Hits?

- Acrylamide, Dieldrin, Heptachlor Epoxide, Phenobarbital, Triethyltin, Bis-n-tributyltin were active in one or two endpoints
- Lack of activity across many endpoints seems to be associated with very slight visual differences in zebrafish behavioral profiles
- How can we potentially assess our confidence in these chemicals as actives in the LMR assay?
  - Binarization of hit/no-hit can be misleading
    - Assessment of concentration response trend across hit and no-hit endpoints for chemicals may indicate evidence, or lack of evidence, of general concentration response behavior
      - Look at quality of non-constant model curve-fits across all endpoint data for a chemical
    - Assessment response intensity in hit and no-hit endpoints for a chemical may reveal intense response poorly fit by curve-fitting procedure
  - Integration of zebrafish LMR data with other DNT NAM data will allow for a better assessment of confidence in chemical's apparent developmental neurotoxicity toxicity

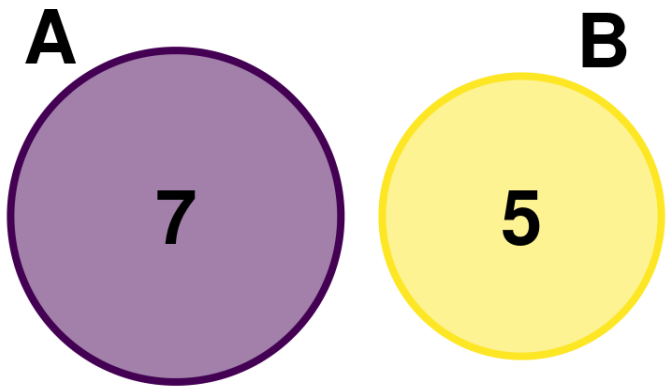
Assay Sensitivity was Only 42%, Why?

- Conservatism of experimental procedure
  - Testing was conducted at concentrations determined not-overtly toxic
  - Zebrafish removed if there was evidence of malformation or death
  - Exposure groups not considered in analysis if rates of malformation or death passed a pre-defined cutoff (25%)
  - One evaluated malformation, swim-bladder inflation, could be evaluated as a behavioral endpoint
    - Zebrafish must manually inflate their swim-bladder
- Criteria used to select chemicals with evidence of *in vivo* DNT
  - Only mammalian studies accessed
    - Multiple species were used across studies (human, rodent, primate)
  - Evidence of DNT came from various DNT outcomes (behavior, morphology, and neurochemistry)
    - Potential that some chemicals selected did not have evidence of effect in *in vivo* behavioral outcomes
  - Selection described in greater detail in Mundy et al. 2015
- Analysis method may be missing chemically induced changes occuring within zebrafish samples
  - Variability amongst individual behavior profiles may increase with chemical exposure

Conclusions

- Assessing the magnitude of zebrafish response to the Light-Dark transition within the locomotor response appears to be informative for assessing the neurological effects of chemicals
- Assessing changes in zebrafish habituation to new light conditions within the Locomotor Response assay appears to be informative for assessing neurological effects of chemicals
- Evaluation of the endpoints presented significantly improved LMR assay sensitivity at a slight reduction in assay specificity
- Evaluation of zebrafish in total distance metrics appears to lose characteristics of the LMR described by the relationship between activity and time

A: Chemicals active in total distance traveled metrics.
B: Chemicals active in Average Acceleration in Dark.



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Questions?

Acknowledgements

Stephanie Padilla

Katie Paul Friedman

Woody Setzer

Richard Judson

Kelly Carstens

Padilla Lab

- Deborah Hunter
- Joan Hedge
- Jeanene Olin
- Kimberly Jarema
- Bridgett Hill
- Bridget Knapp
- Katy Britton
- Morgan Lowery

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Supplemental Slides

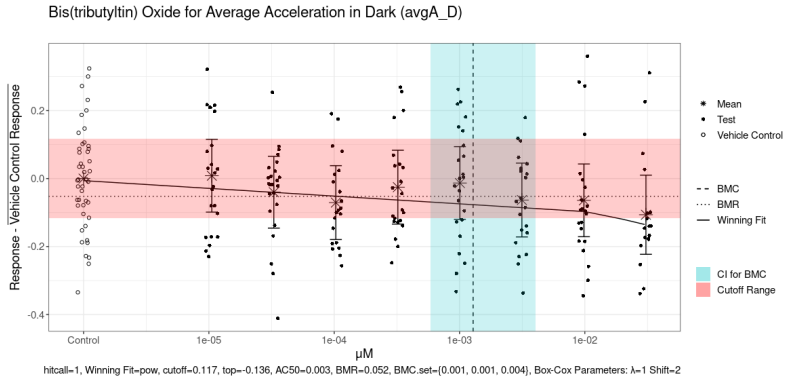
High Confidence and Low Confidence Hits

High Confidence Hits	Low Confidence Hits
5,5-Diphenylhydantoin	5-Fluorouracil
6-Aminonicotinamide	Acrylamide
Amphetamine	Bis(tributyltin) Oxide
BPA	Cocaine Base
Chlorpyrifos (ethyl)	Diazepam
Fluoxetine	Dieldrin
Heptachlor	Diethylstilbesterol
Paraquat	D-sorbitol
PBDE-47	Heptachlor epoxide
Tebuconazole	Loperamide
	Phenolbarbital
	Triethyltin

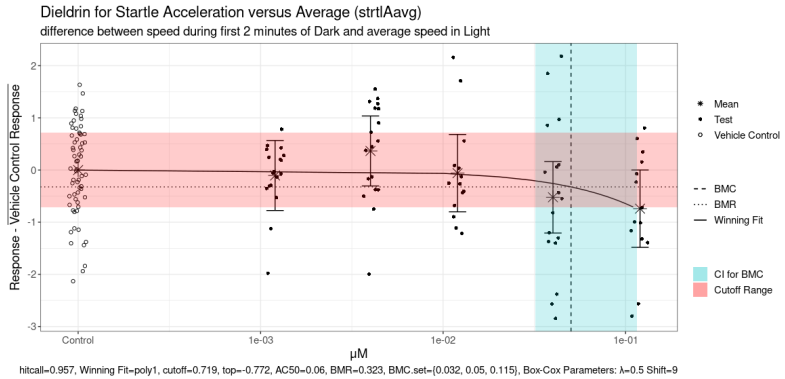
High Confidence Hits are active in many endpoints and/or have well-fitting curve-fits to concentration response data. Low Confidence Hits are not active in many endpoints, have curve fits to the concentration response data that are poor, and/or are considered putative DNT negatives.

Chemicals Found Active in One Endpoint

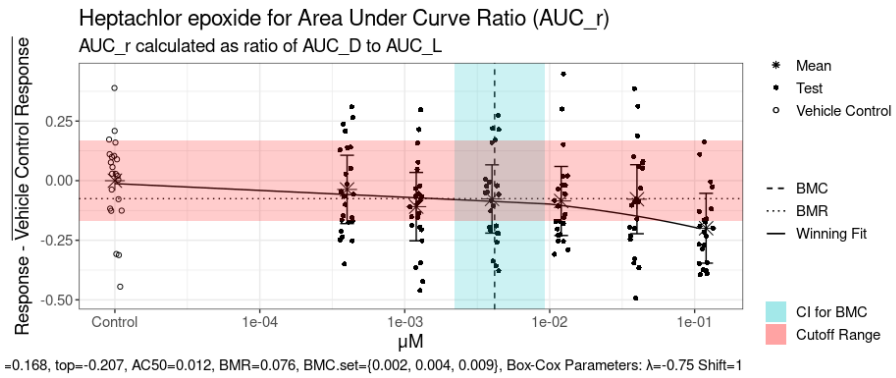
Bis(tributyltin) Oxide



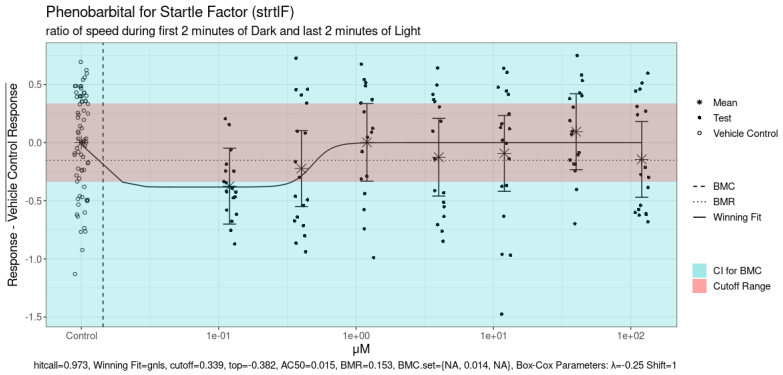
Dieldrin



Heptachlor epoxide



Phenobarbital



Triethyltin