EPA United States Environmental Protection Agency

Novel in vitro Methods for Ecological Species: Evaluating Cross-species Differences in Nuclear Receptor-Ligand Interactions

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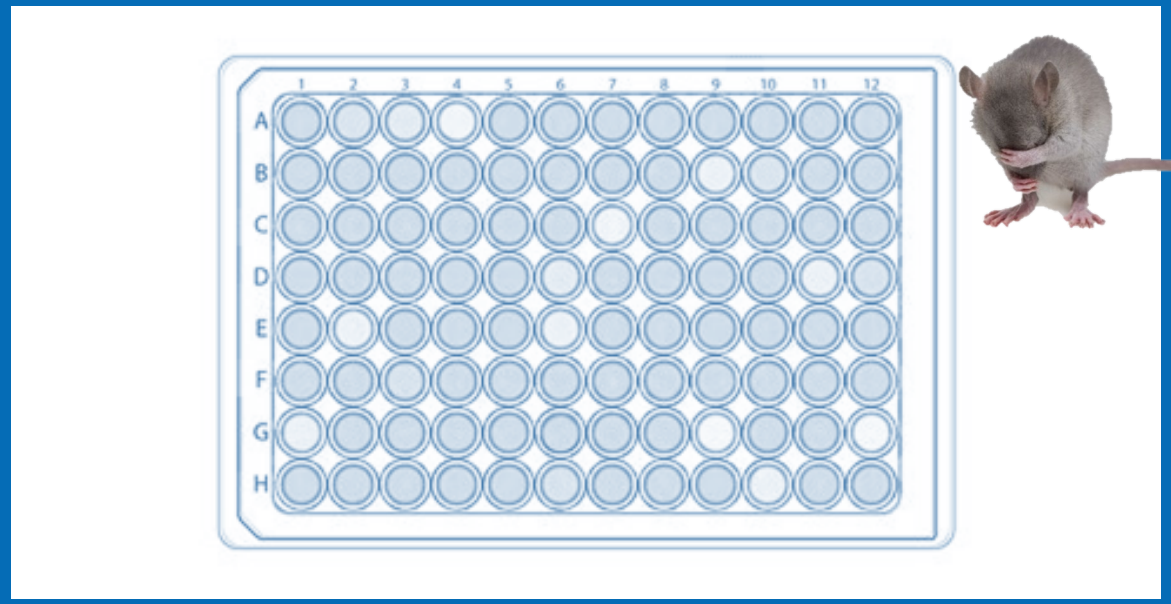
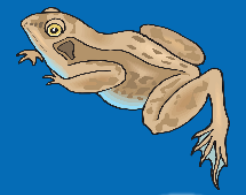
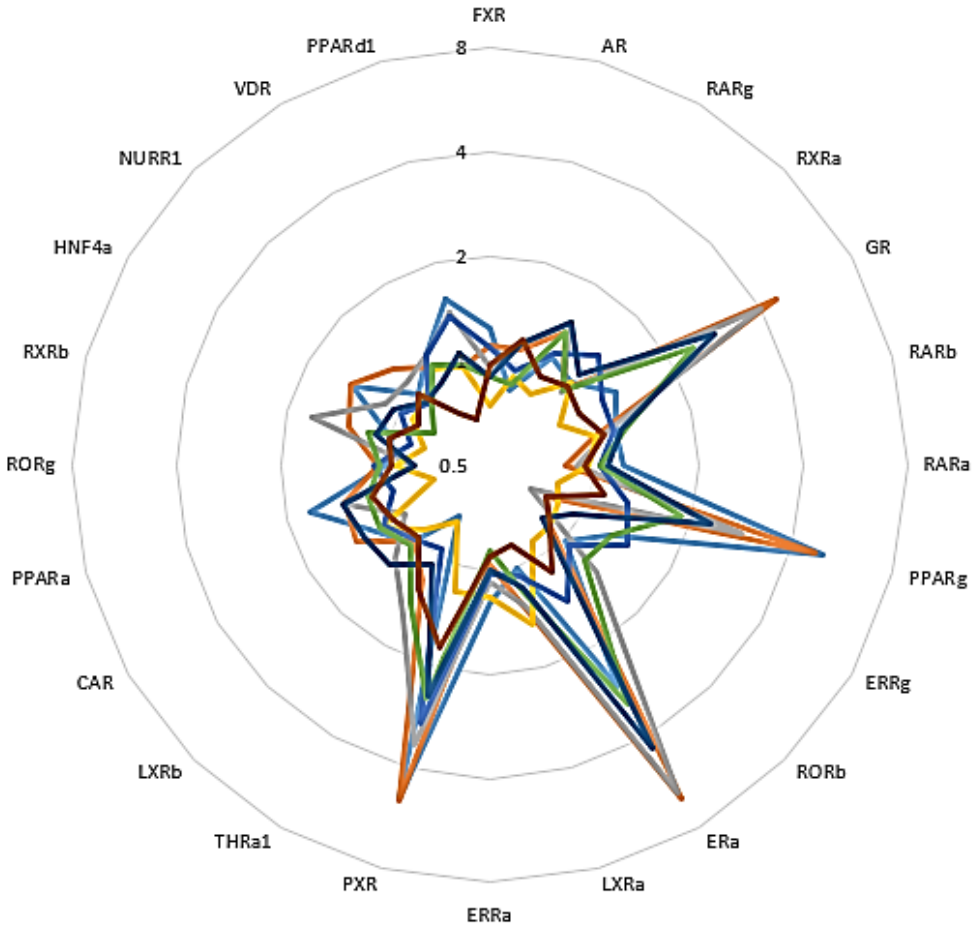




Office of Research and Development
Center for Computational Toxicology and Exposure, Great Lakes Toxicology and Ecology Division

EPA at 50 Progress for a Stronger Future

Environmental Monitoring with Attagene TRANS-FACTORIAL Assay



- PXR
- ERα
- PPARγ
- GR
- PPARα
- RXRβ

Do the human receptors adequately represent sensitivity of aquatic vertebrate receptors?

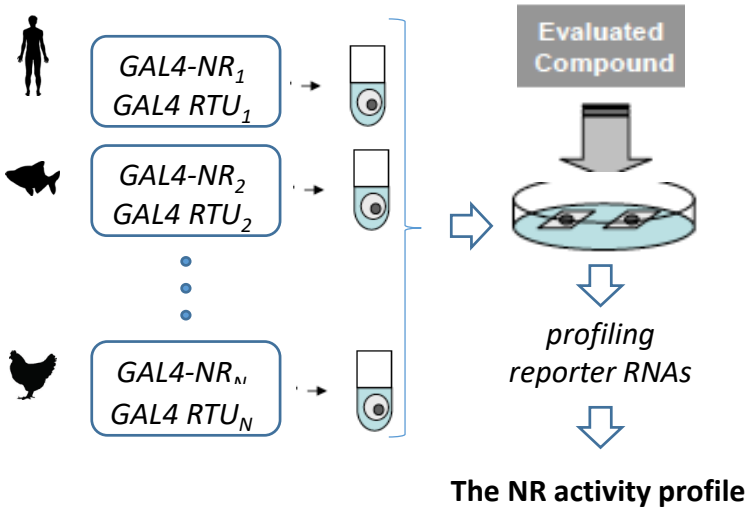
Among the most frequently detected nuclear receptor activities in surface water samples

Cross-species extrapolation

- To date, high throughput screening has been human centric
- Unclear how well mammalian HTS assays represent vertebrate diversity, let alone other phyla.
- Not feasible to include all taxa in a HTS screening program.

How can we strategically select the minimum number of representative species that cover the maximal range of variation in sensitivity and specificity?

Attagene EcoTox FACTORIAL Assay



NR	Class	Species	Sequence ID
ER1	Fish	*Danio rerio*	NM_152959.1
ERα		*Danio rerio*	NM_180966.2
ERβ		*Danio rerio*	NM_174862.3
ER1	Amphibian	*Xenopus laevis*	NM_001089617
ER2		*Xenopus laevis*	NM_001130954
ER1	Reptilian	*Chrysemys picta*	NM_001282246
ER1	Avian	*Gallus gallus*	NM_205183
ERα	Mammalian	*Homo Sapiens*	NM_000125
ERβ		*Homo Sapiens*	NM_001437
AR	Fish	*Danio rerio*	NM_001083123
AR	Amphibian	*Xenopus laevis*	NM_001090884
AR	Reptilian	*Chrysemys picta*	XM_005279527
AR	Avian	*Gallus gallus*	NM_001040090
AR	Mammalian	*Homo Sapiens*	NM_000044
TRα	Fish	*Danio rerio*	NM_131396.1
TRβ		*Danio rerio*	NM_131340.1
TRα	Amphibian	*Xenopus laevis*	NM_001088126
TRα	Reptilian	*Chrysemys picta*	XM_005294120
TRα	Mammalian	*Homo Sapiens*	NM_199334
TRβ		*Homo Sapiens*	NM_000461
PPARγ	Fish	*Danio rerio*	NM_131467
PPARγ	Mammalian	*Mus musculus*	NM_001127330
PPARγ		*Homo Sapiens*	BC006811
PXR	Mammalian	*Mus musculus*	NM_010936

Considered 5 vertebrate classes
Focused on endocrine NRs

Differences in sensitivity among vertebrate classes were generally minor for ER, AR, TR.

Fish PPARγ was substantially less sensitive to classic PPARγ agonists than mammals.

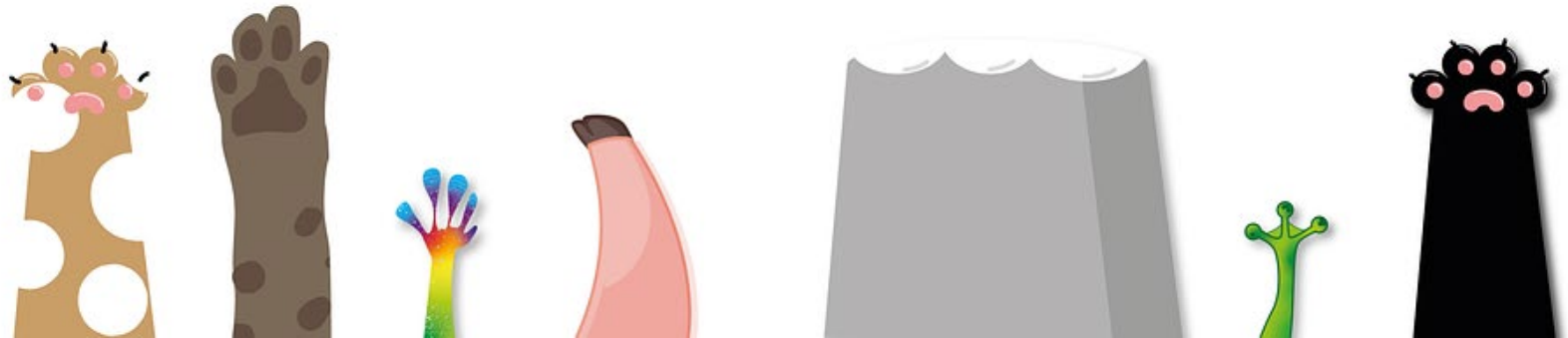
*Medvedev et al. Harmonized cross-species assessment of endocrine and metabolic disruptors by EcoTox FACTORIAL assay. Environmental Science & Technology **2020** 54 (19), 12142-12153. DOI: 10.1021/acs.est.0c03375.*

Species Selection

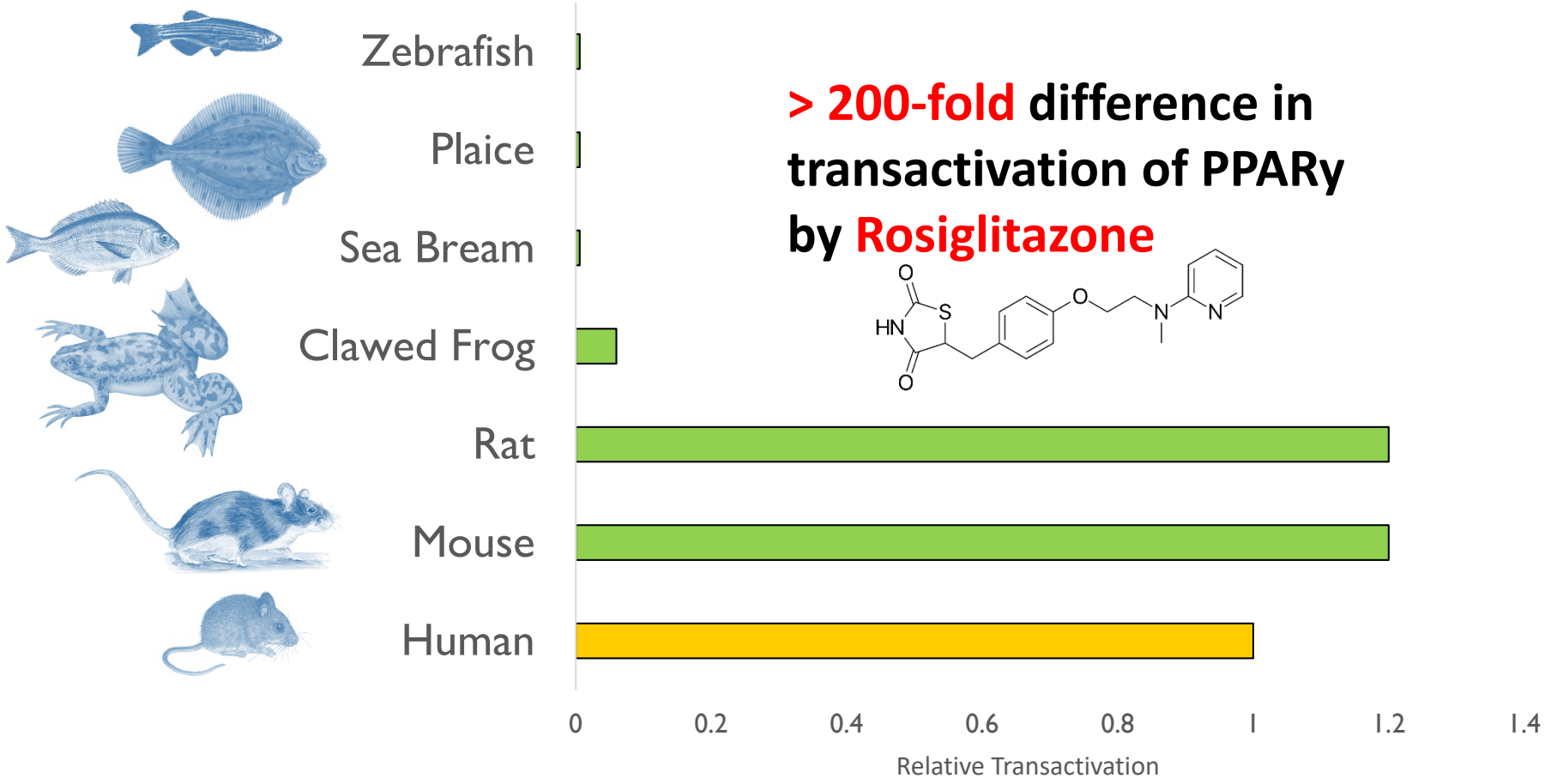
Is the selection of one representative vertebrate from each class the best way to cover the potential variability in sensitivity?

Could available information be used to guide a more strategic selection?

- Documented species differences in sensitivity to ligands
- Amino acid residues identified as critical to ligand binding in one or more species
- In silico analyses of conservation/variation in aa sequence using SeqAPASS



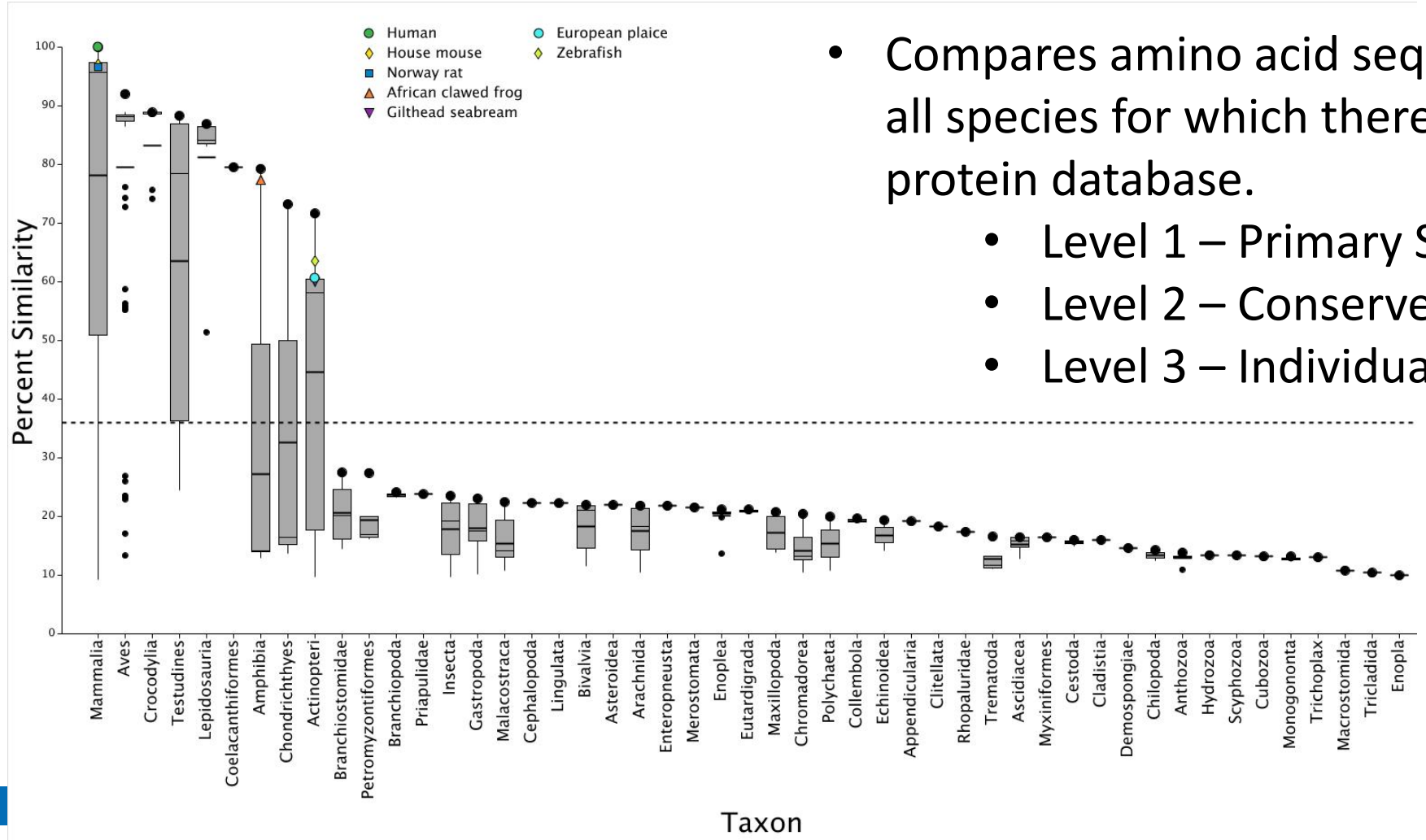
PPARγ – established cross-species differences



> 200-fold difference in transactivation of PPARγ by Rosiglitazone

SeqAPASS Analysis

https://seqapass.epa.gov/seqapass/



- Compares amino acid sequence information for all species for which there are data in the NCBI protein database.
  - Level 1 – Primary Sequence
  - Level 2 – Conserved domains
  - Level 3 – Individual amino acid residues

Example SeqAPASS Level 3 - PPARγ

- Only 4 positions showed important differences in amino acids among PPARγ
- 2 positions known to significantly alter interaction of ligand (rosiglitazone) with PPARγ

Taxa	# of Species	Position 1 (Ile309)	Position 2 (Gly312)	Position 3 (Cys313)	Position 4 (Tyr355)	Susceptibility Prediction
Human	1	I	G	C	Y	Yes
All Mammals	107	I	G	C	Y	Yes
Mallard-type	1	I	G	C	Y	Yes
Most Birds	70	I	R	C	Y	No
All Reptiles	19	I	R	C	Y	No
All Amphibians	2	I	R	C	F	No
Ancient Fishes	9	F	R	C	I	No
Most Fishes	61	F	S	C	I	No
Salmonid-type	3	V	R	I	T	No
Bonytongue-type	1	F	R	W	I	No
Zebrafish-type	2	F	S	Y	I	No

Strongly conserved among most birds, amphibians, reptiles

More variation among various orders of fishes than across other vertebrate classes

Example SeqAPASS Level 3 - PPARγ

- *In silico* mechanism for lack of Rosiglitazone binding to zebrafish PPARγ is severe steric hindrance from Gly312Ser and Cys313Tyr mutation
- Comparing positions 312 and 313 of human to other species

Taxa	Species	Position 312	Position 313	Susceptibility Prediction	Relative Transactivation
Mammal	Human	G	C	Yes	1.0
Mammal	Mouse	G	C	Yes	1.2
Mammal	Rat	G	C	Yes	1.2
Amphibian	Clawed Frog	R	C	No	0.06
Fish	Sea Bream	S	C	No	<0.006
Fish	Plaice	S	C	No	<0.006
Fish	Zebrafish	S	Y	No	<0.006

Strategic Approach

Similar types of analyses applied to

GR

PPARα

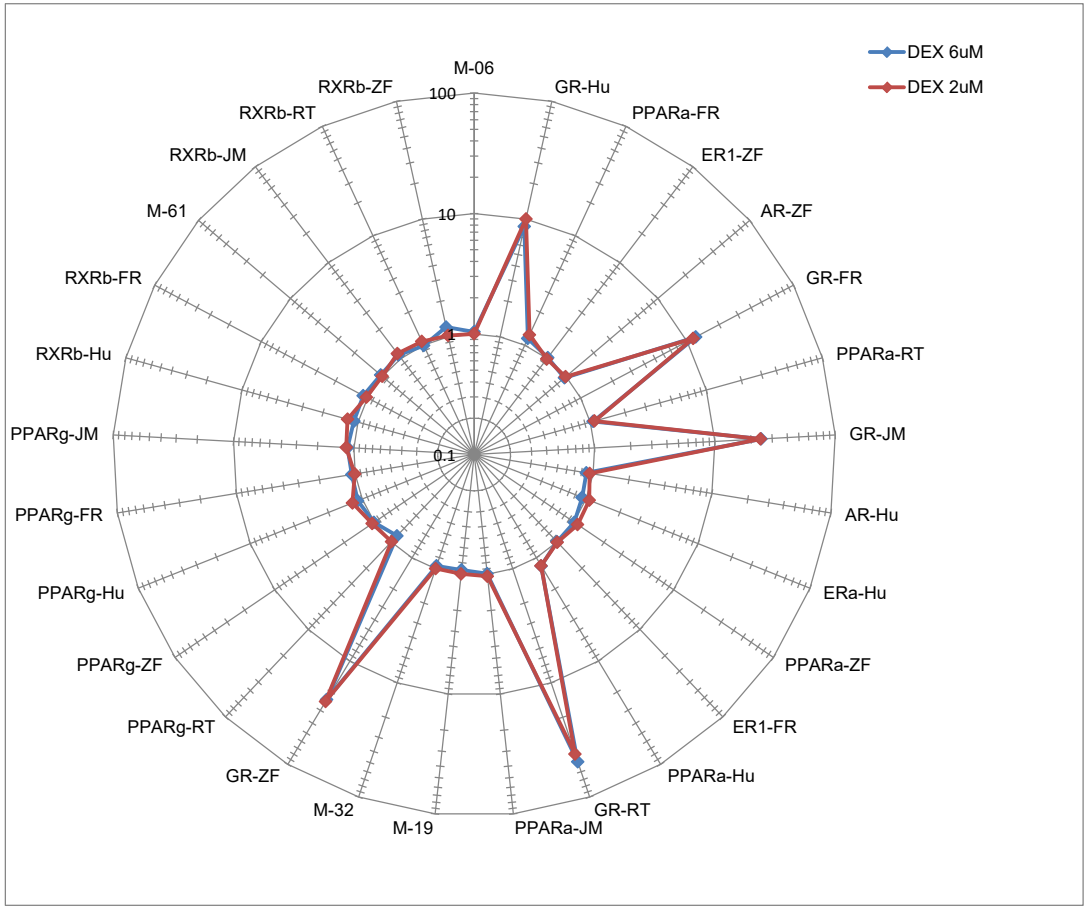
RXRb

Selected a group of species that should capture maximum diversity in response for these four NRs (& genomes available)

- Human
- Xenopus laevis
- Rainbow trout
- Japanese medaka
- Zebrafish

Attagene EcoTox-2 Factorial assay

#	Name	Species	Latin names
1	GR	human	Homo Sapiens
2	GR	african clawed frog	Xenopus laevis
3	GR	rainbow trout	Oncorhynchus mykiss
4	GR	japanese medaka	Oryzias latipes
5	GR	Zebrafish	Danio rerio
6	PPARa	human	Homo Sapiens
7	PPARa	african clawed frog	Xenopus laevis
8	PPARa	rainbow trout	Oncorhynchus mykiss
9	PPARa	japanese medaka	Oryzias latipes
10	PPARa	Zebrafish	Danio rerio
11	PPARg	human	Homo Sapiens
12	PPARg	african clawed frog	Xenopus laevis
13	PPARg	rainbow trout	Oncorhynchus mykiss
14	PPARg	japanese medaka	Oryzias latipes
15	PPARg	Zebrafish	Danio rerio
16	RXRb	human	Homo Sapiens
17	RXRb	african clawed frog	Xenopus laevis
18	RXRb	rainbow trout	Oncorhynchus mykiss
19	RXRb	japanese medaka	Oryzias latipes
20	RXRb	Zebrafish	Danio rerio
21	ERa	human	Homo Sapiens
22	ER1	Zebrafish	Danio rerio
23	ER1	african clawed frog	Xenopus laevis
24	AR	human	Homo Sapiens
25	AR	Zebrafish	Danio rerio

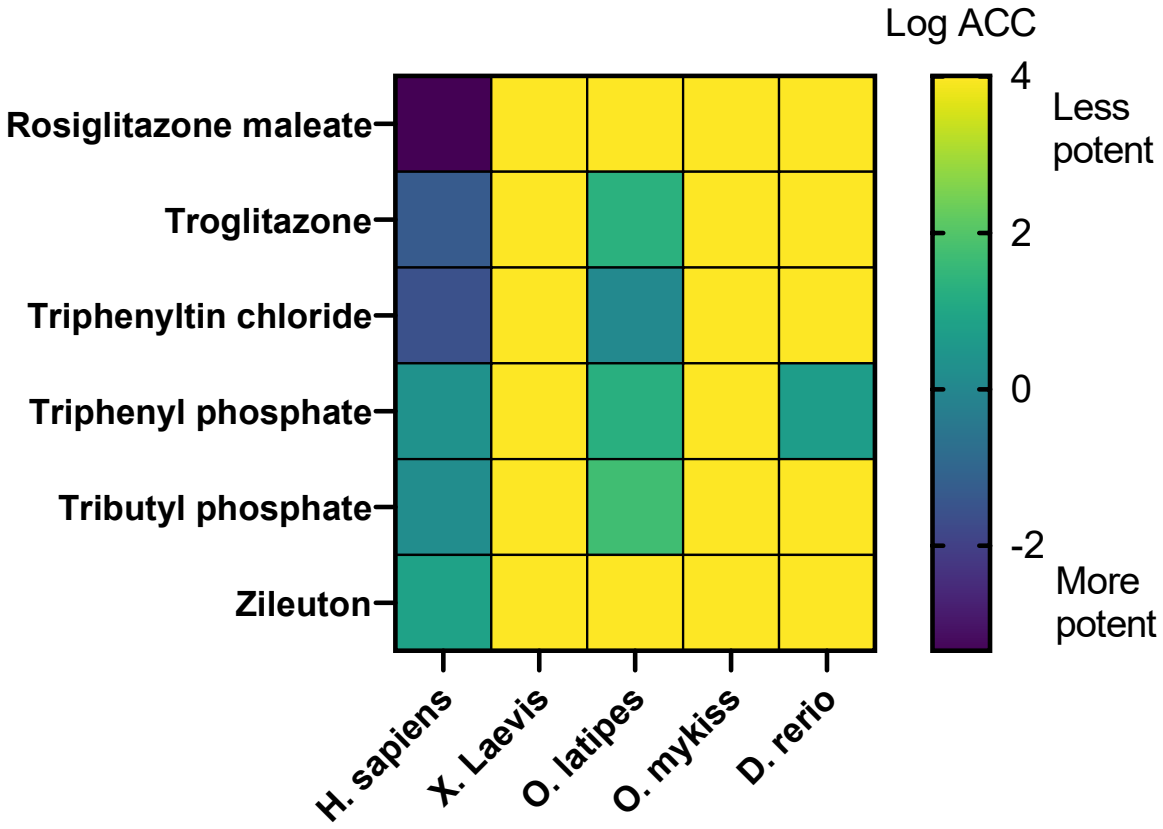


- 14 chemicals in concentration-response
- Surface water extracts

Test Chemicals

Test Chemical	Target
Rosiglitazone maleate	PPARg
Tributyl phosphate	PPARg, PXR
Prednisone	GR, AR
Troglitazone	PPARg, PPARa
Zileuton	PPARg; ALOX5
Bexarotene	RxRb
Gemfibrozil	PPARa
Butachlor	GR, AR (env)
Triphenyl phosphate	PPARg (env)
Fenofibrate	PPARa
Dexamethasone NaPO4	GR
Triphenyltin chloride	RxR, RAR
PFOA	PPARa (env)
Potassium PFHxS	PPARa (env)

Results - PPARγ

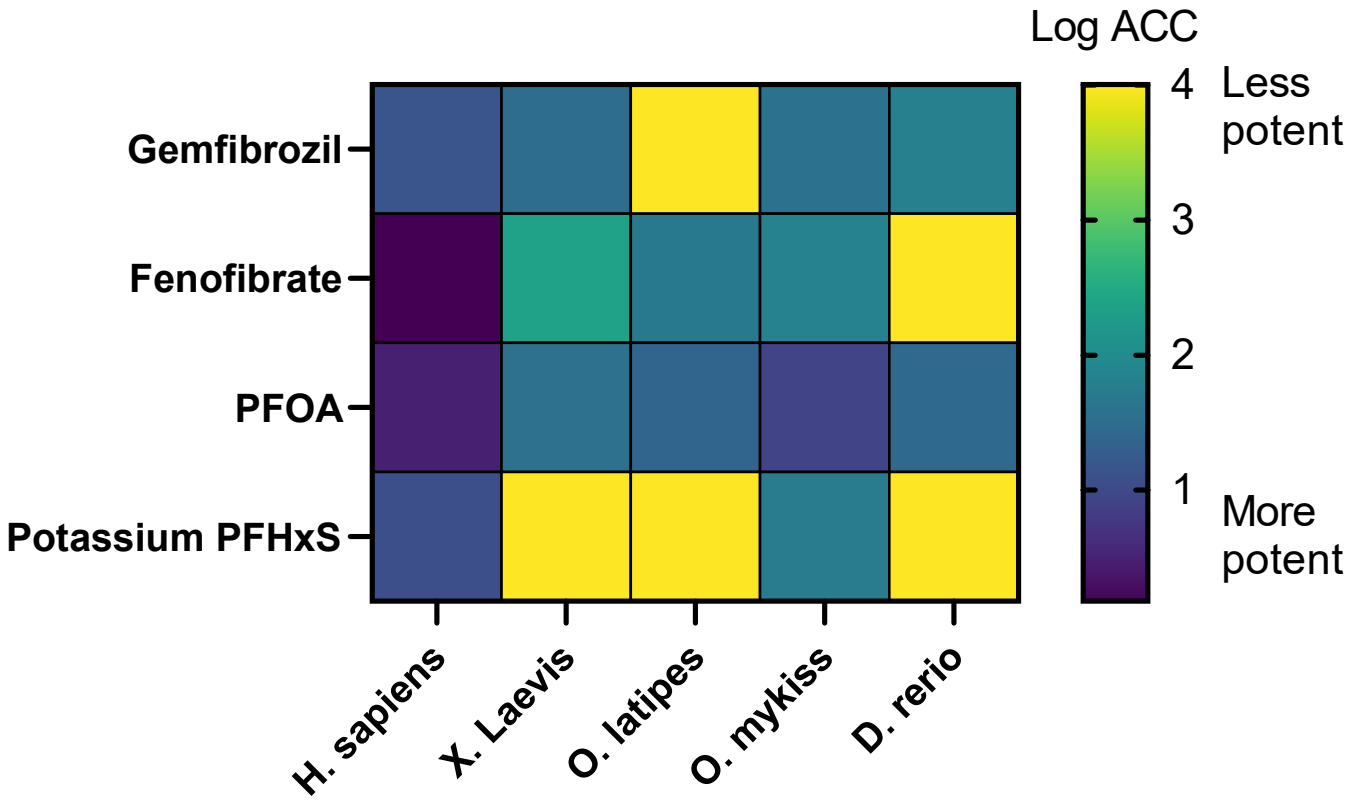


Predicted Susceptible

Taxa	Human (H. sapiens)	Frog (X. laevis)	Medaka (O. latipes)	Trout (O. mykiss)	Zebrafish (D. rerio)
Rosiglitazone	Y 1.0	N 0.06	N <0.006	N <0.006	N <0.006

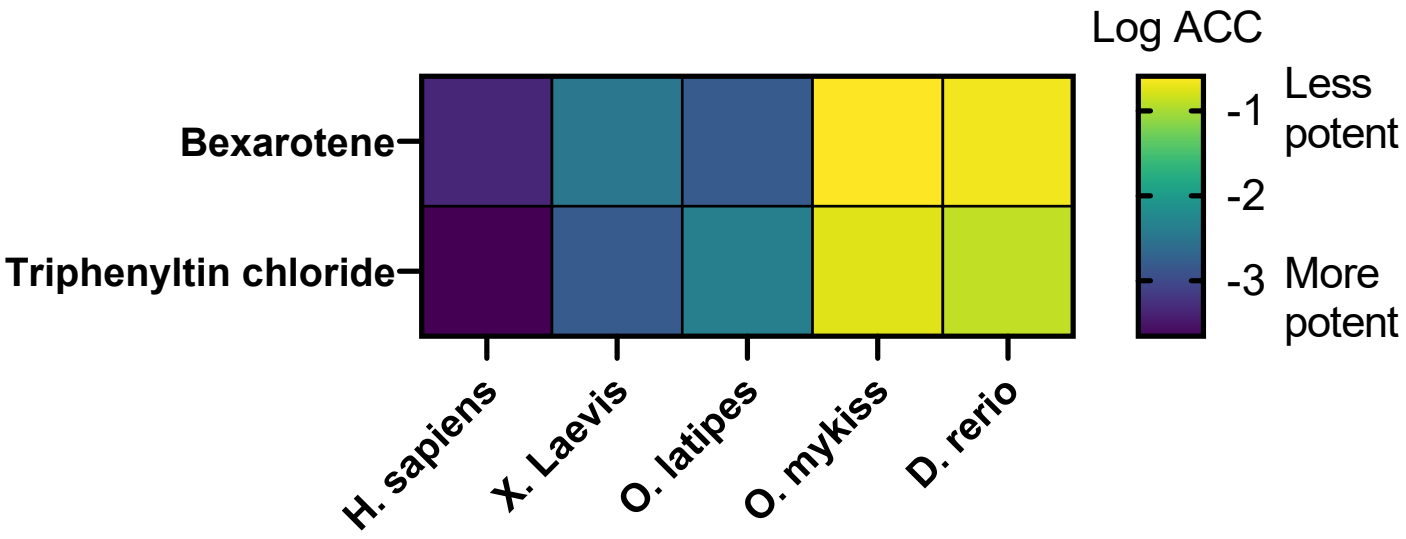
- As predicted, only human PPARγ was sensitive to rosiglitazone
- Among the other PPARγ agonists, Xenopus and rainbow trout were insensitive
- Japanese medaka, selected to represent “most fishes” showed partial sensitivity to some, but not all ligands.
- Zebrafish were sensitive to TPP, but not other ligands

Results - PPARα



- Medaka PPARα was insensitive to gemfibrozil
- Zebrafish PPARα was insensitive to fenofibrate
- Results suggest that aa residues critical to binding gemfibrozil and fenofibrate may differ
- All species sensitive to PFOA
- Human and rainbow trout demonstrated sensitivity to PFHxS

Results - RXRβ

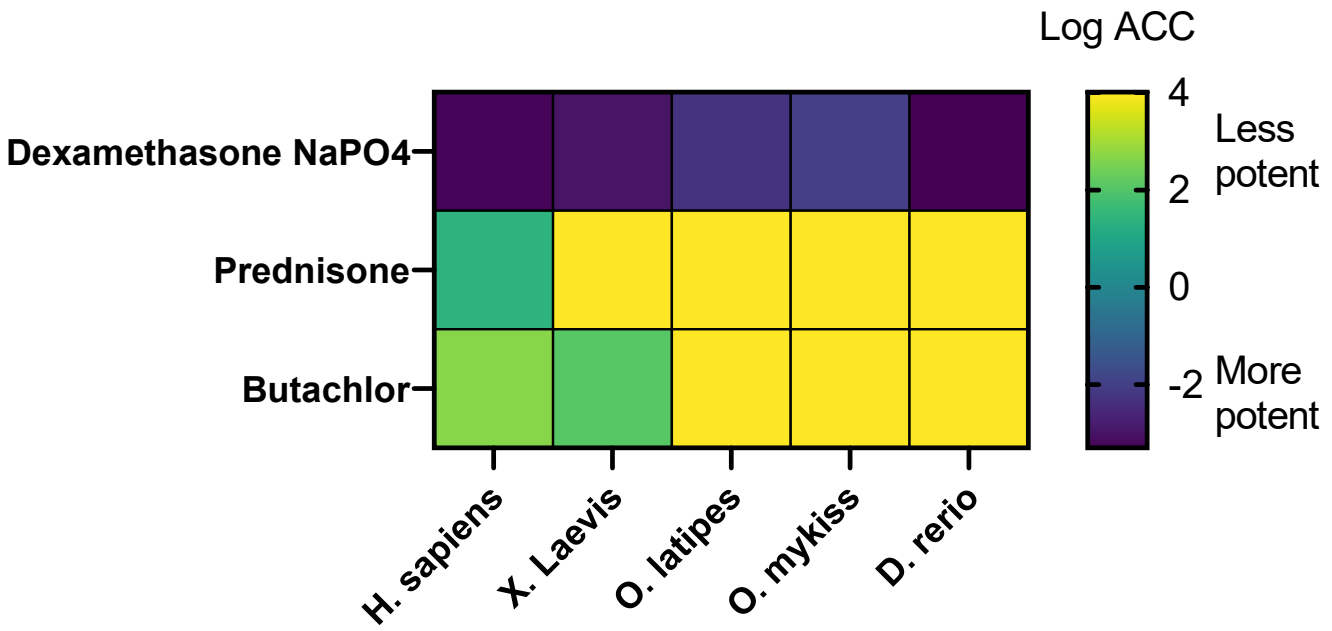


- Rainbow trout and zebrafish RXRb were less sensitive to RXRb ligands than the other species tested.

Results - GR

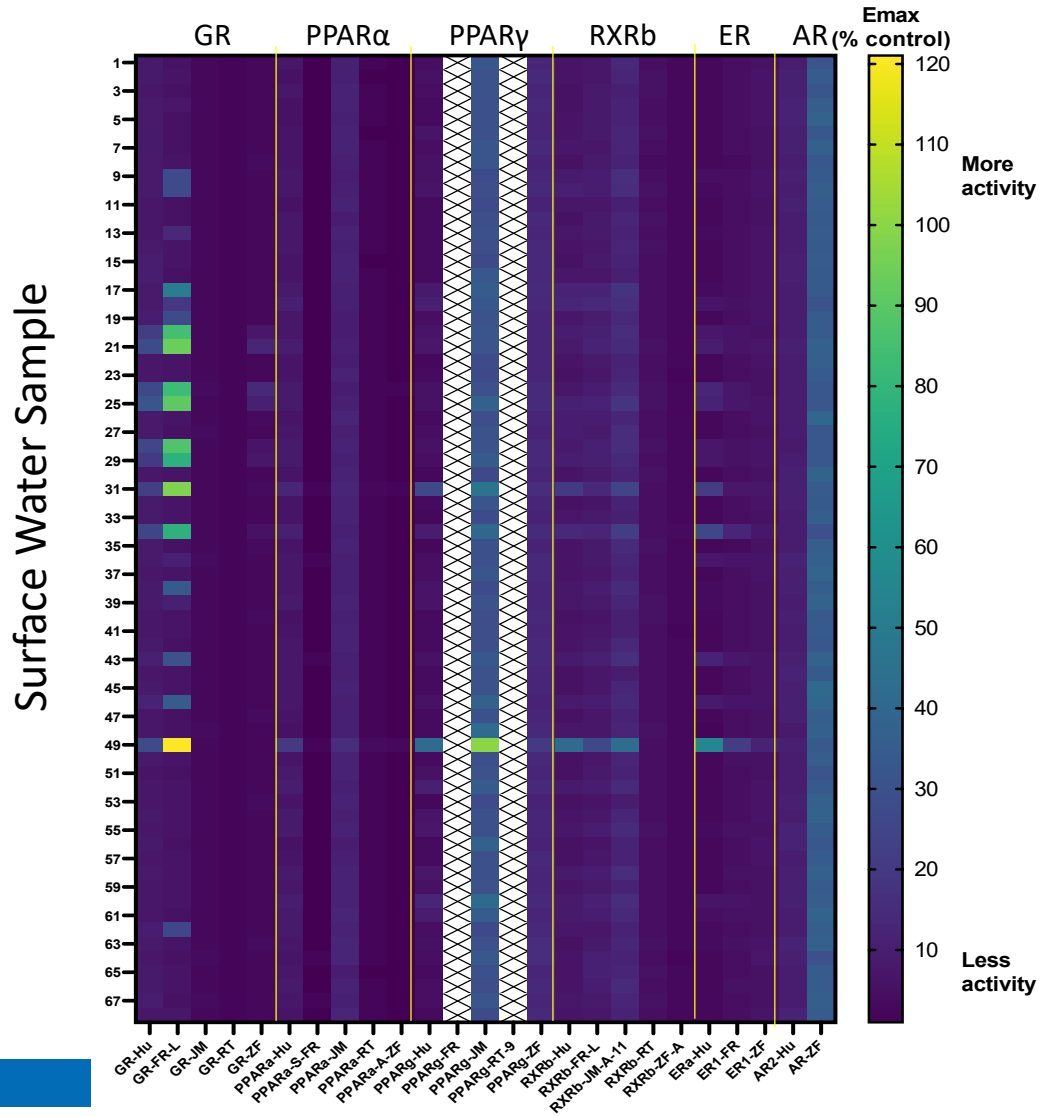
Predicted Susceptible

Taxa	Homo sapiens	Xenopus laevis	Oryzias latipes	Oncorhynchus mykiss	Danio rerio
Dexamethasone	Y	Y	N	N	Y



- Predictions were qualitatively accurate for dexamethasone but reflected different sensitivity, not overall susceptibility
- Need to metabolically activate prednisone to the GR-active prednisolone complicates interpretation

Application to Environmental Monitoring



- 68 surface water samples screened
- Among the GRs, Xenopus GR was the most responsive to GR-active compounds in environmental mixtures
- Among the PPARγ Japanese medaka PPARγ was the most responsive to the environmental mixtures
- Samples with the greatest activity were consistently elevated in all species, proportional to their intrinsic relative sensitivity.

Conclusion

- Effects-based monitoring employing human cell lines using human nuclear receptors (hNR) are likely to yield different conclusions than if fish NRs were employed (at least for PPARγ, PPARα, RXRβ, and GR).

- Variations among different orders of fish may be as substantial as across other classes of vertebrates.

- Different chemical-specific profiles across species were consistent with a previous assumption that level 3 SeqAPASS analyses based on specific ligand-chemical interactions may not apply universally across relevant chemical space.
  - Complicates the ability to select a minimum number of species to capture maximum variability in sensitivity.

- Screening of additional chemicals using the XS-2 Factorial Assay may yield new insights that improve the ability to predict cross-species susceptibility based on aa sequence.

References

- *Cavallin et al. Effects-Based Monitoring of Bioactive Chemicals Discharged to the Colorado River before and after a Municipal Wastewater Treatment Plant Replacement. Environmental Science & Technology 2021, 55, 2, 974–984. https://doi.org/10.1021/acs.est.0c05269*
- *Lalone et al. Sequence Alignment to Predict Across Species Susceptibility (SeqAPASS): A Web-Based Tool for Addressing the Challenges of Cross-Species Extrapolation of Chemical Toxicity. Toxicological Sciences, Volume 153, Issue 2, October 2016, Pages 228–245, https://doi.org/10.1093/toxsci/kfw119*
- *Medvedev et al. Harmonized cross-species assessment of endocrine and metabolic disruptors by EcoTox FACTORIAL assay. Environmental Science & Technology 2020 54, 19, 12142-12153. https://doi.org/10.1021/acs.est.0c03375*