

Epigenetic effects of environmentally relevant concentrations of estrogens in multiple lifestages of the fathead minnow (*Pimephales promelas*)

Adam Biales, Chief, Molecular Indicators Branch, Office of Research and Development, US EPA



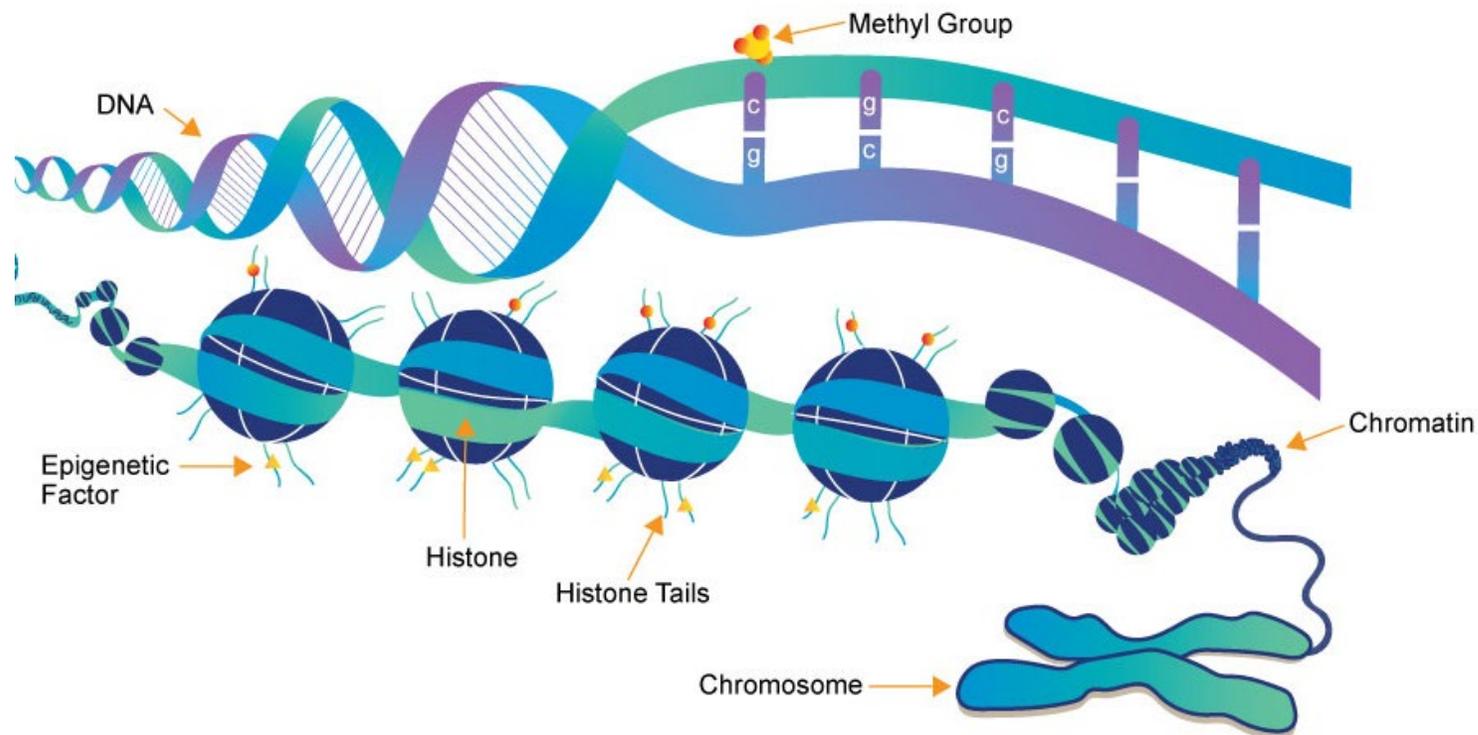
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Overview

- Epigenetics
- Regulatory implications
- Environmental estrogens
- Early life stage exposure
 - small non-coding RNA
- Adult exposure
 - ESR1
 - Genome/epigenome-wide



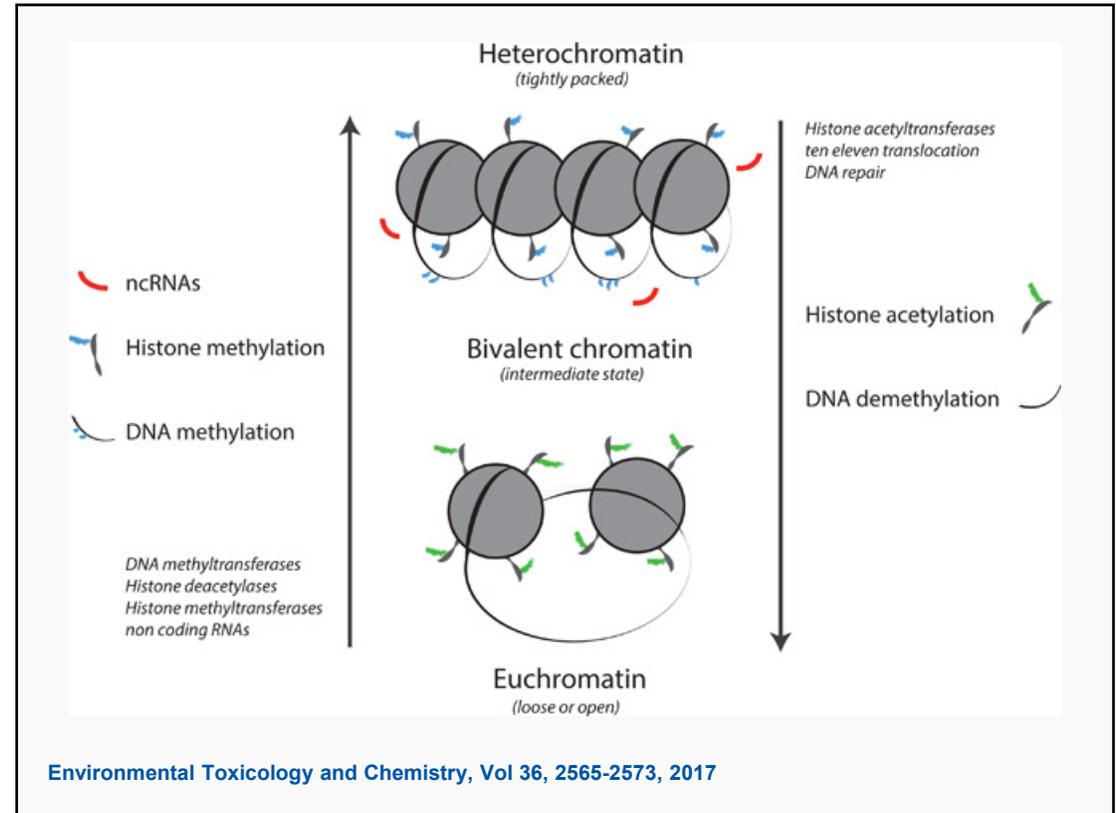
Epigenetics



- Heritable phenotypic changes
 - No change in underlying DNA sequence
- Adaptation
 - Genome X Environment
 - Relevant time scale
- Implicated in disease

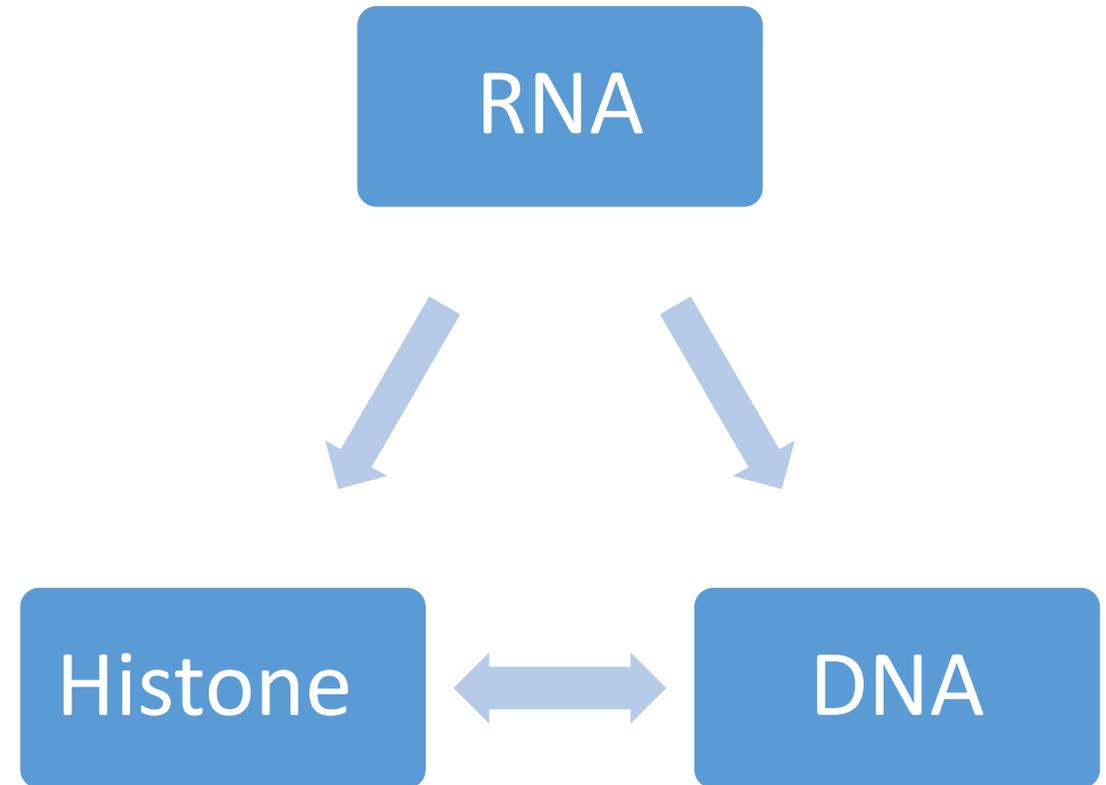
Epigenetics

- Mechanisms
 - Gene expression levels
 - Chromatin state
 - sncRNA
 - DNA methylation
 - Histone modification

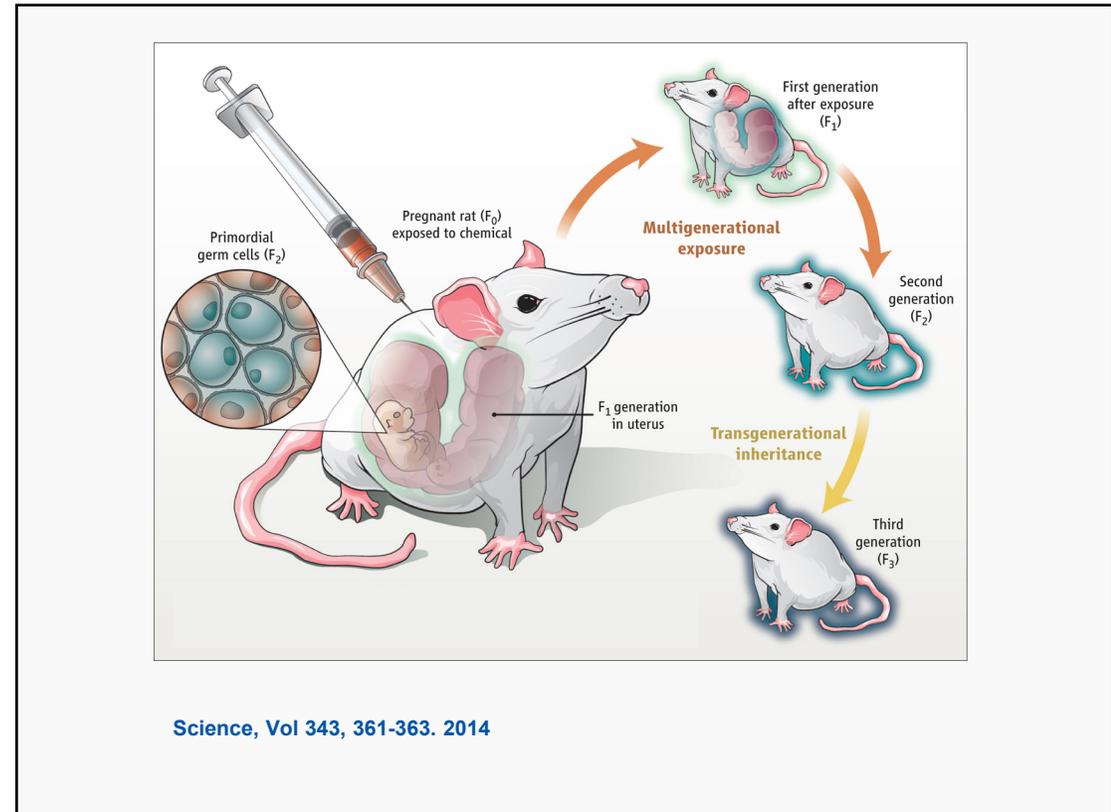


Epigenetic mechanism

- Work in concert
 - ncRNA
 - miRNA - ↓ methyltransferases
 - piRNA – guide → DNA methylation
 - DNA methylation
 - CpGs
 - CpG islands and shores
 - Promoter vs. gene body ↑↓
 - Histone modification
 - Methylation
 - Acetylation



- Heritable
 - Mitotic
 - Meiotic
- Trans/Multigenerational effects
 - Phenotypic changes
 - Disease
 - Vinclozolin -> ↑ ovarian cancer
 - Metabolic disease



Epigenetics in environmental regulation



- Susceptibility
 - Genes related to pharmacokinetics
 - CYPs, transporters, etc.
- Multigenerational effects
 - Sublethal effects
 - Ecosystem function
- Predictive biomarkers
 - Adverse effects
- Forensic biomarkers
 - Different time scales
- Exposome

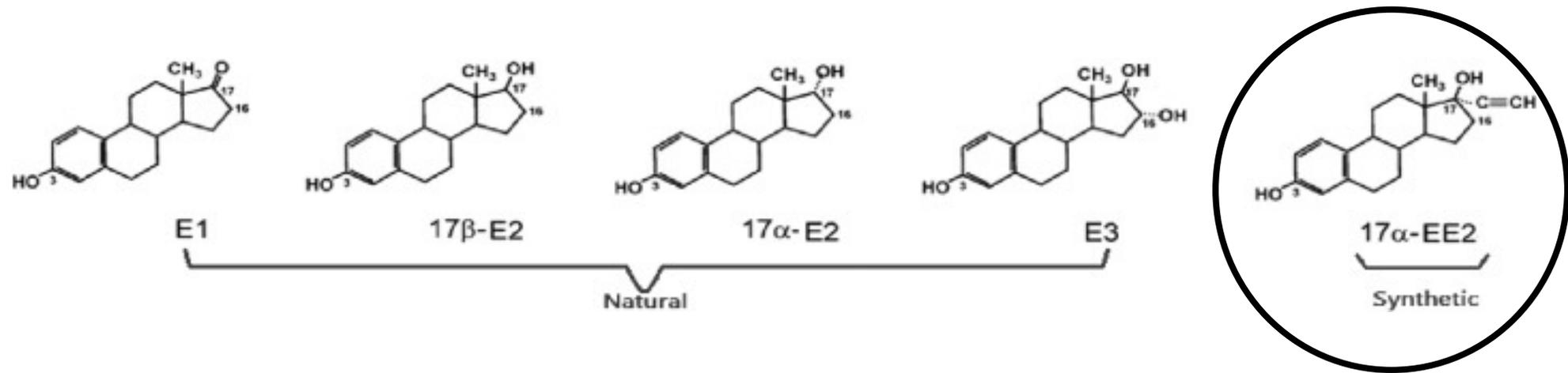
Fathead minnow

- Commonly used aquatic toxicity model for N. America
- Endemic – huge natural range
- Highly contiguous and complete genome

De novo assembly and annotation of a highly contiguous reference genome of the fathead minnow (*Pimephales promelas*) reveals an AT-rich repetitive genome with compact gene structure

John Martinson¹, David C. Bencic¹, Gregory P. Toth¹, Mitchell S. Kostich^{1,3}, Robert W. Flick¹, Mary J. See¹, David Lattier¹, Adam D. Biales^{1*}, Weichun Huang^{2*}

Assembly statistics	FHM1 (GCA_000700825)	FHM2 (WIOS000000000)	ZF (GRCz11)
Number of scaffolds	73,057	910	993
N50 contig	7,513	300,151	1,428,257
N50 scaffold	60,380	11,952,773	54,304,671
Complete BUSCOs	3,506 (76.5%)	4,357 (95.1%)	4,384 (95.7%)
Complete and single-copy BUSCOs	3,324 (72.5%)	4,115 (89.8%)	4,215 (92.0%)
Complete and duplicated BUSCOs	182 (4.0%)	242 (5.3%)	169 (3.7%)
Fragmented BUSCOs	507 (11.1%)	73 (1.6%)	66 (1.4%)
Missing BUSCOs	571 (12.4%)	153 (3.3%)	134 (2.9%)
Total contig size	811,183,656	925,375,343	1,368,782,359
Total scaffold size	1,219,326,373	1,066,412,313	1,373,471,384



Environmental Estrogens

- Identified intersex individuals
- Experimental Lake Study -> population collapse
- Commonly found in low ng/L to pg/L in surface water
 - Biologically active levels
- Mixtures – additive effects
- Difficult to predict estrogenicity based on structure

Small Non-coding RNA

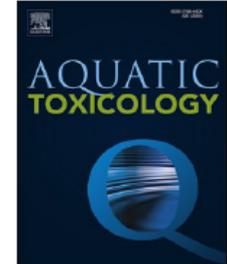


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Aquatic Toxicology

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Development of omics biomarkers for estrogen exposure using mRNA, miRNA and piRNAs

Gregory P. Toth^a, David C. Bencic^a, John W. Martinson^a, Robert W. Flick^a, David L. Lattier^a, Mitchell S. Kostich^b, Weichun Huang^c, Adam D. Biales^{a,*}

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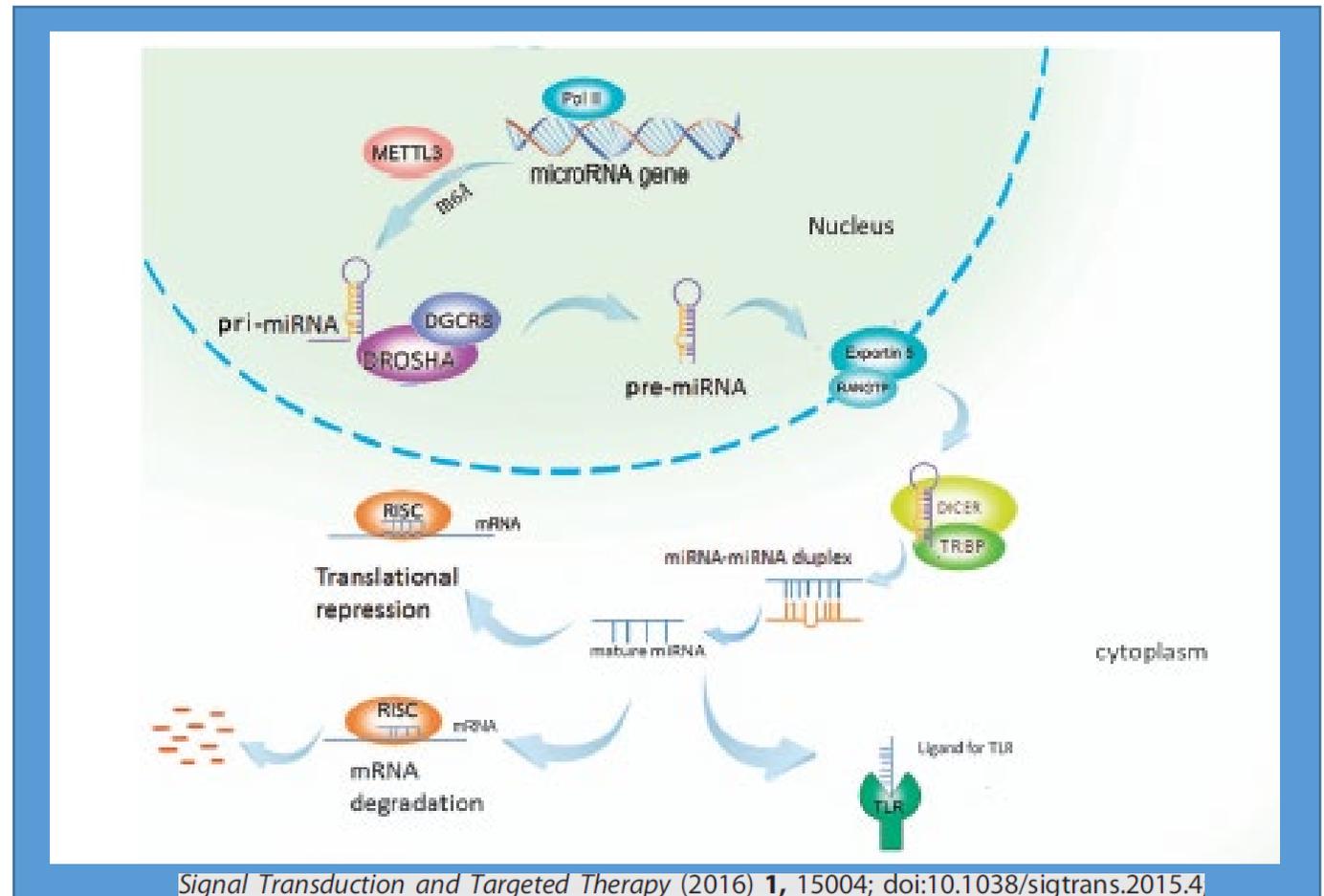


Biomarkers development

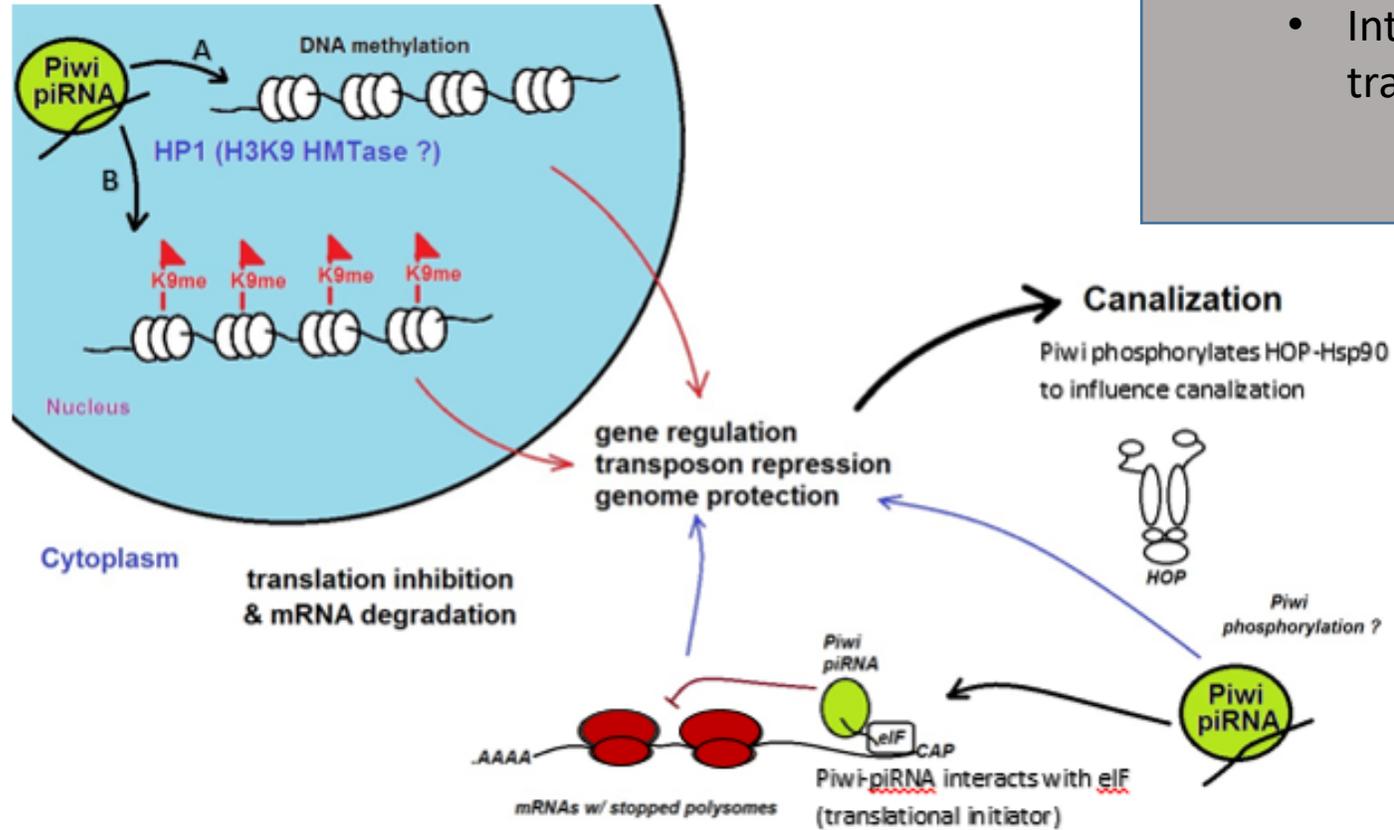
- Evaluate biomarker training and testing scenarios
 - Increase effective concentration range
- Evaluate sncRNAs as potential source of biomarkers
 - Implicated in broad number of diseases
 - One to many -> smaller number of biomarkers
 - Extracellular
- Evaluate genome assembly and annotation
 - 620 FHM miRNAs miRDeep – compare to *Danio* miRNAs for exact matches
 - piRNA -> mapped against *Danio* piRNA reference set 1.33M

MicroRNA - Mechanism

- RISC – AGO proteins
- Translational repression
- mRNA degradation



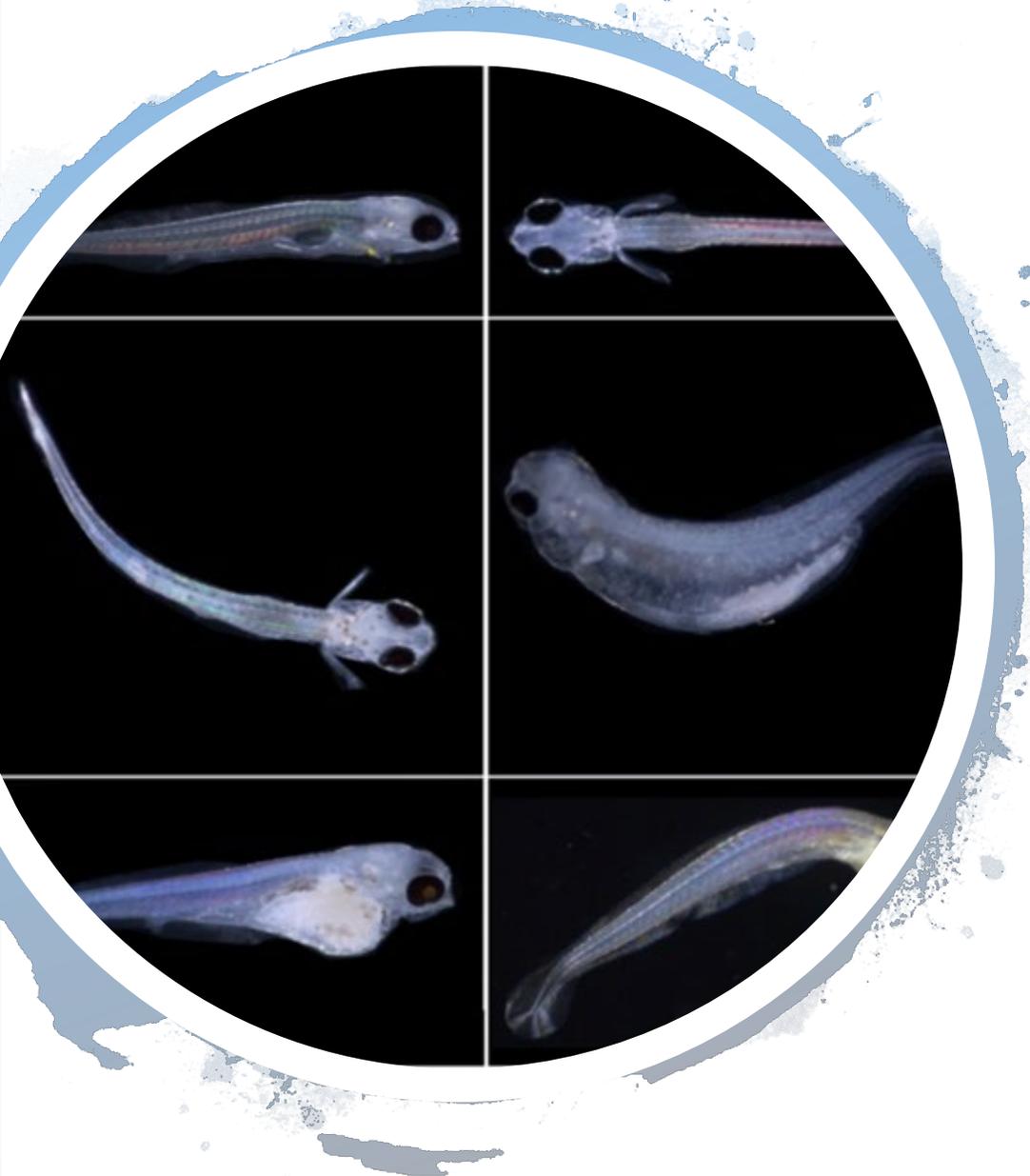
Piwi-interacting RNA



Mechanisms

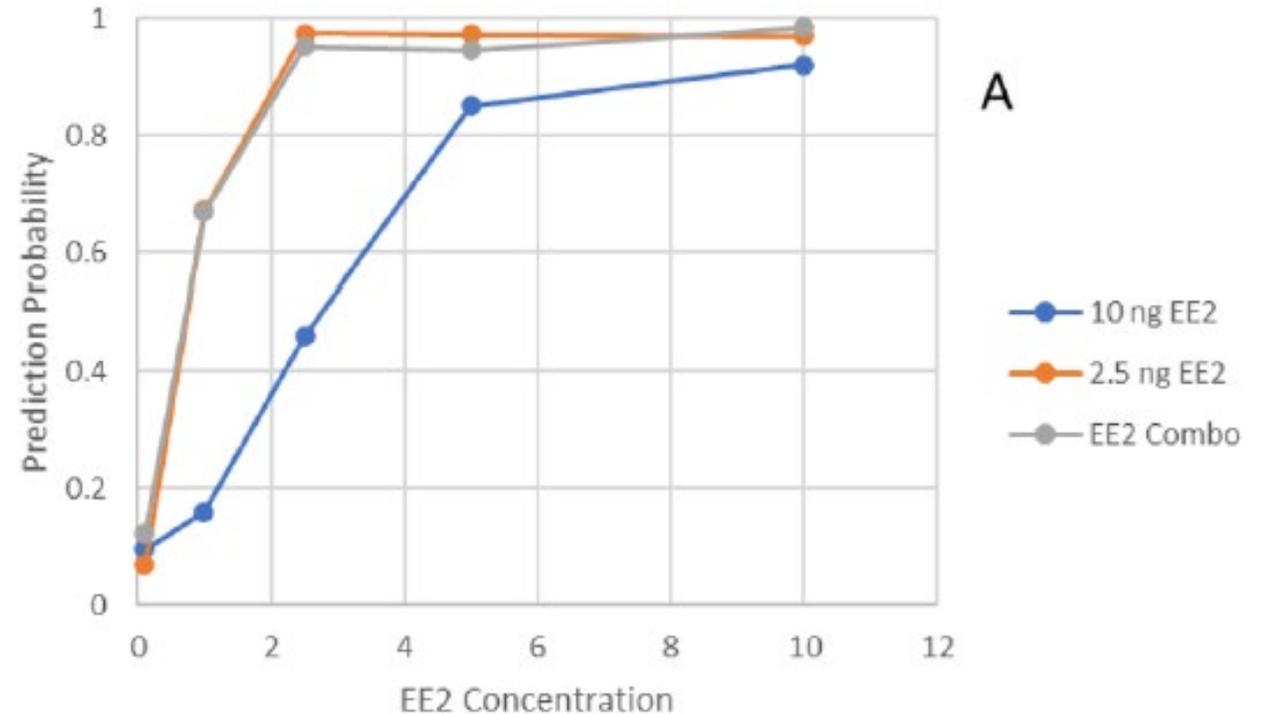
- Transcriptional silencing
 - Methylation
- Translational silencing
 - Interaction with translational machinery

Design



- Exposure
 - Three identical experiments
 - 96 hpf – 48 h exposure
 - 0.12 – 10 ng/L EE2
- mRNA
 - Sense RNA-seq 1 X 50 bp SE – HiSeq 4000
 - n = 30 per treatment from across experiments (control, 1.2, 10 ng/L)
 - N = 18 (.12, 2.5, 5 ng/L)
- sncRNA
 - TruSeq small RNA kit
 - n = 10 per treatment (control vs. 10 ng/L EE2)

- mRNA
 - Differential expression in all but lowest concentration
 - Near complete overlap
 - Circadian related transcripts
 - Few typical estrogen-related genes
 - Esr1
 - Vtg1
 - aromatase
- 23 miRNA and 12 piRNA – none were significant after FDR
 - Random Forest classification
 - **miRNA - AUC 0.83**
 - **piRNA – AUC 1.0**



Summary

- mRNA classifier accurate at environmental/biologically active concentrations
- sncRNAs potential for biomarker development
 - Relatively high accuracy
 - sncRNA induced at biologically relevant concentrations of EE2
 - Potential for indicators of timing
- Mechanistic interpretation difficult
 - Poorly annotated
 - One-to-many regulation & poor sequence complementarity
 - Whole larvae

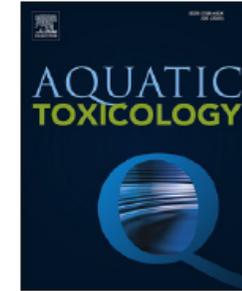


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Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox



DNA methylation and expression of estrogen receptor alpha in fathead minnows exposed to 17 α -ethynylestradiol

J.K. Fetke^{a,d}, J.W. Martinson^b, R.W. Flick^b, W. Huang^c, D.C. Bencic^b, M.J. See^b, E.M. Pilgrim^b, R.W. Debry^d, A.D. Biales^{b,*}

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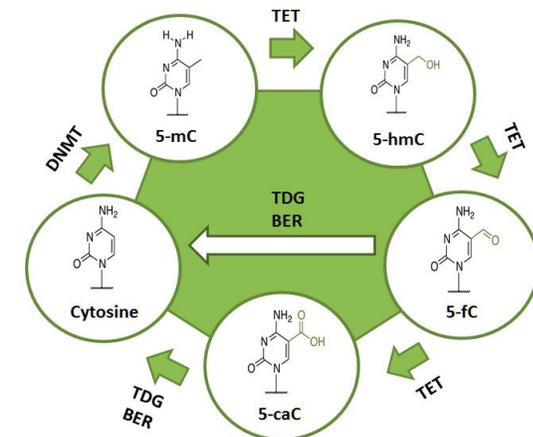
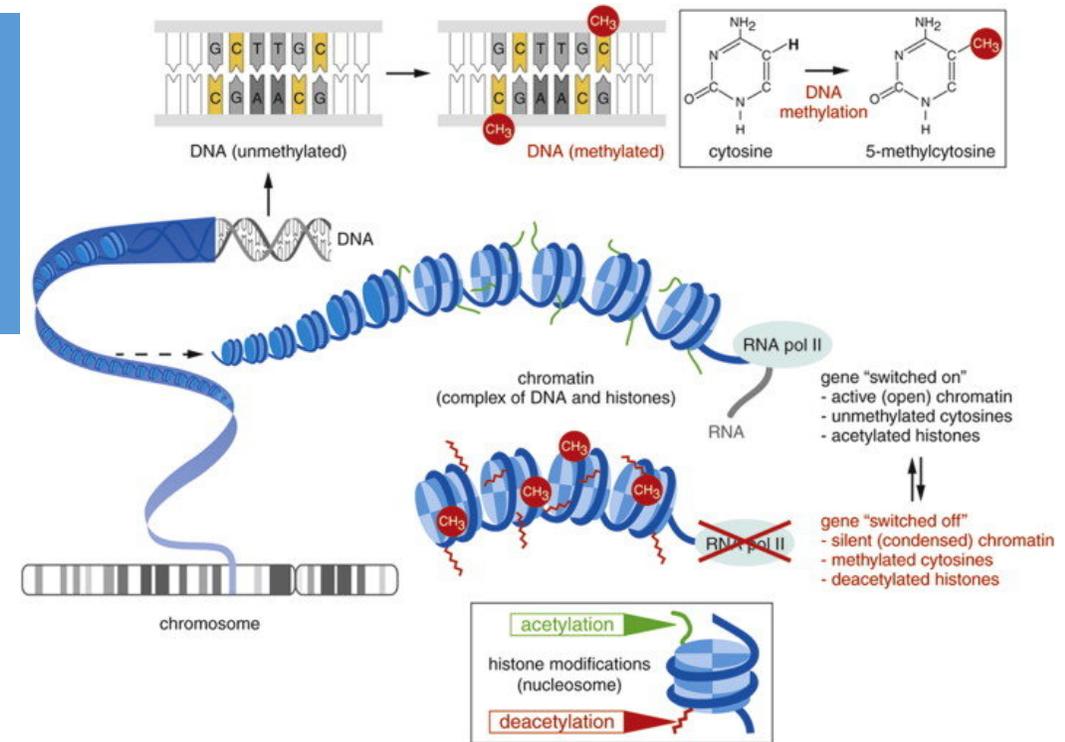
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^d Department of Biological Sciences, University of Cincinnati, Cincinnati, OH, United States

Methylation

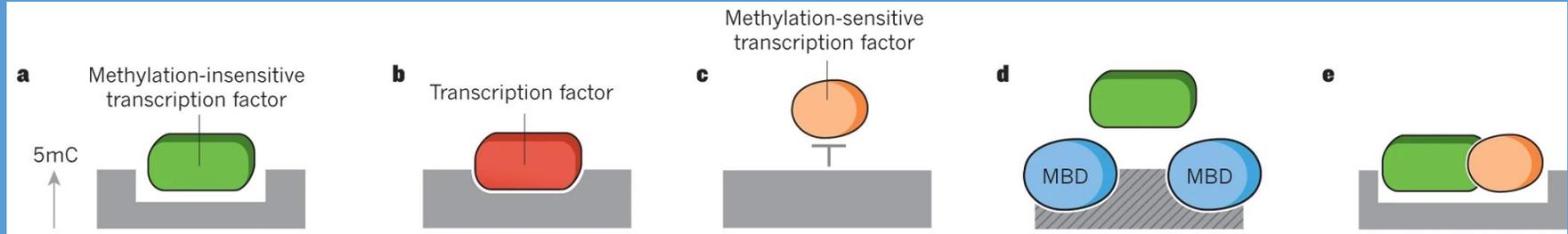
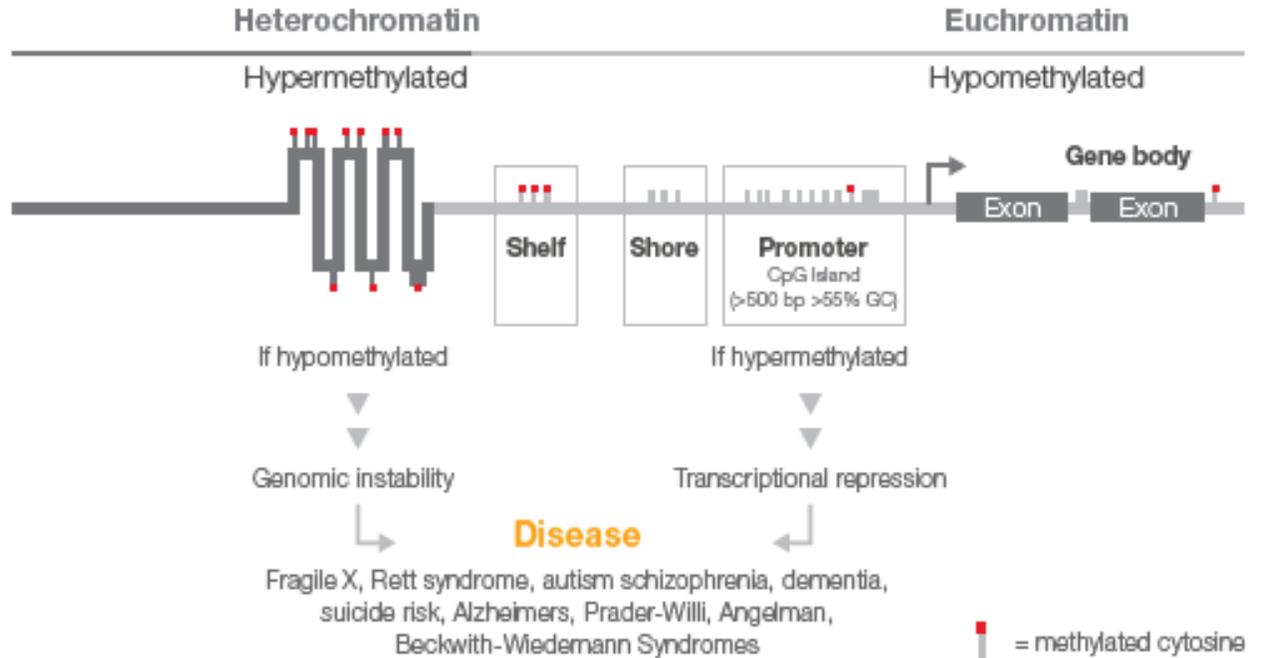
- Experimentally accessible
- Mostly in CpG context in animals
 - 60-90% in mammals, < in inverts
 - Differs across taxa (*D. melanogaster*, *C. elegans*)
- DNMTs
 - DNMT1 – maintenance
 - DNMT3a & b – do novo
- Demethylation – passive
 - TET - active



DNA Methylation Mechanism/Function

- Mechanism
 - Chromatin state
 - Methyl-sensitive TFs
- Promoter (CGI)
 - Unmethylated
 - Tx repression
- Gene Body
 - Highly methylated
 - Increased Tx
 - Exon usage
 - Alt TSS
- Shore – correlate to Tx level
- Shelf – unclear
- Open Sea

Perturbation of Methylation



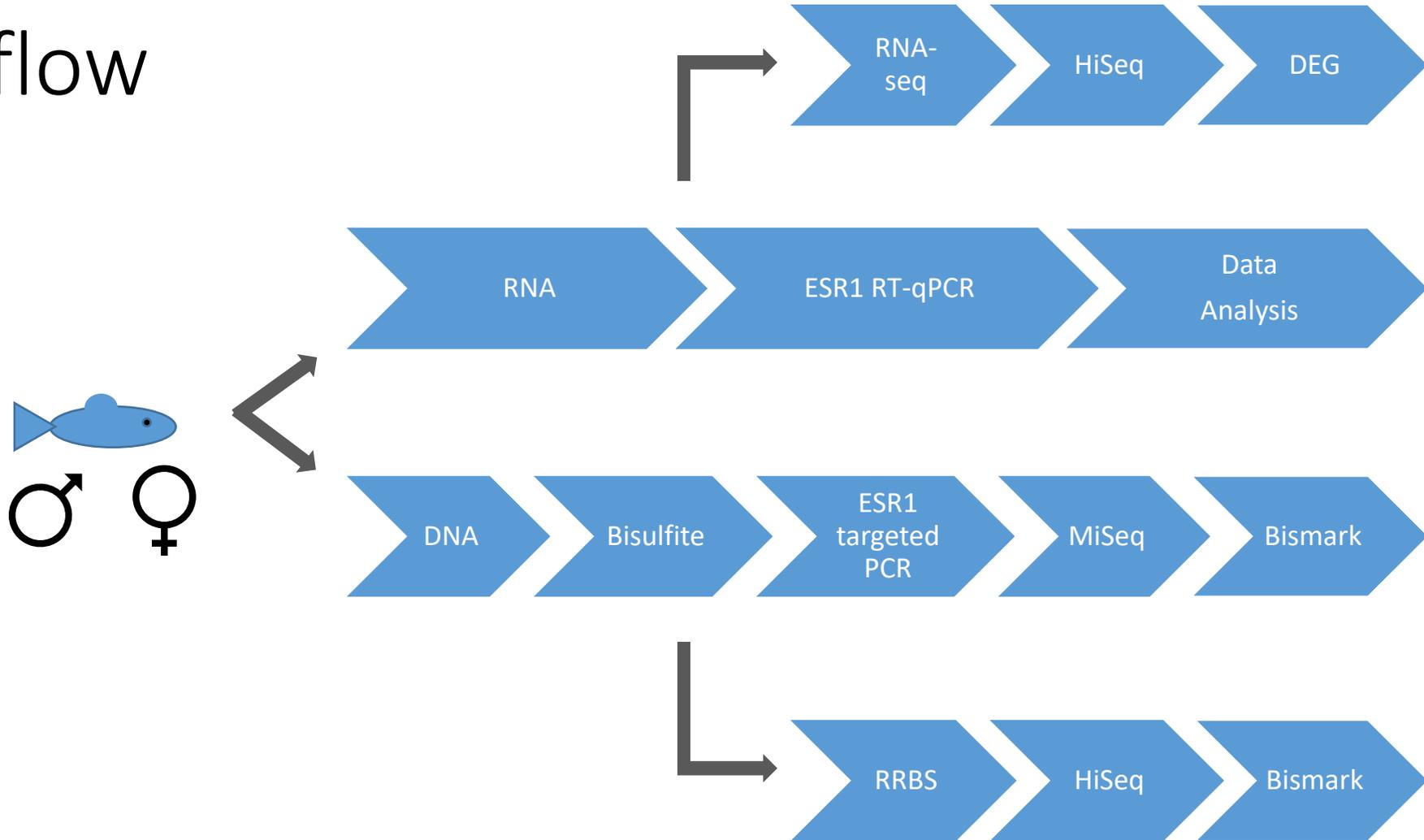
Study Design

Depuration

	Day 2	Day 7	Day 14
0 ng/L	n=27/16	n=8	n=9
2.5 ng/L	n=27/16	n=9	n=10
10 ng/L	n=29/16	n=8/8	n=8/8
Females	n=8		

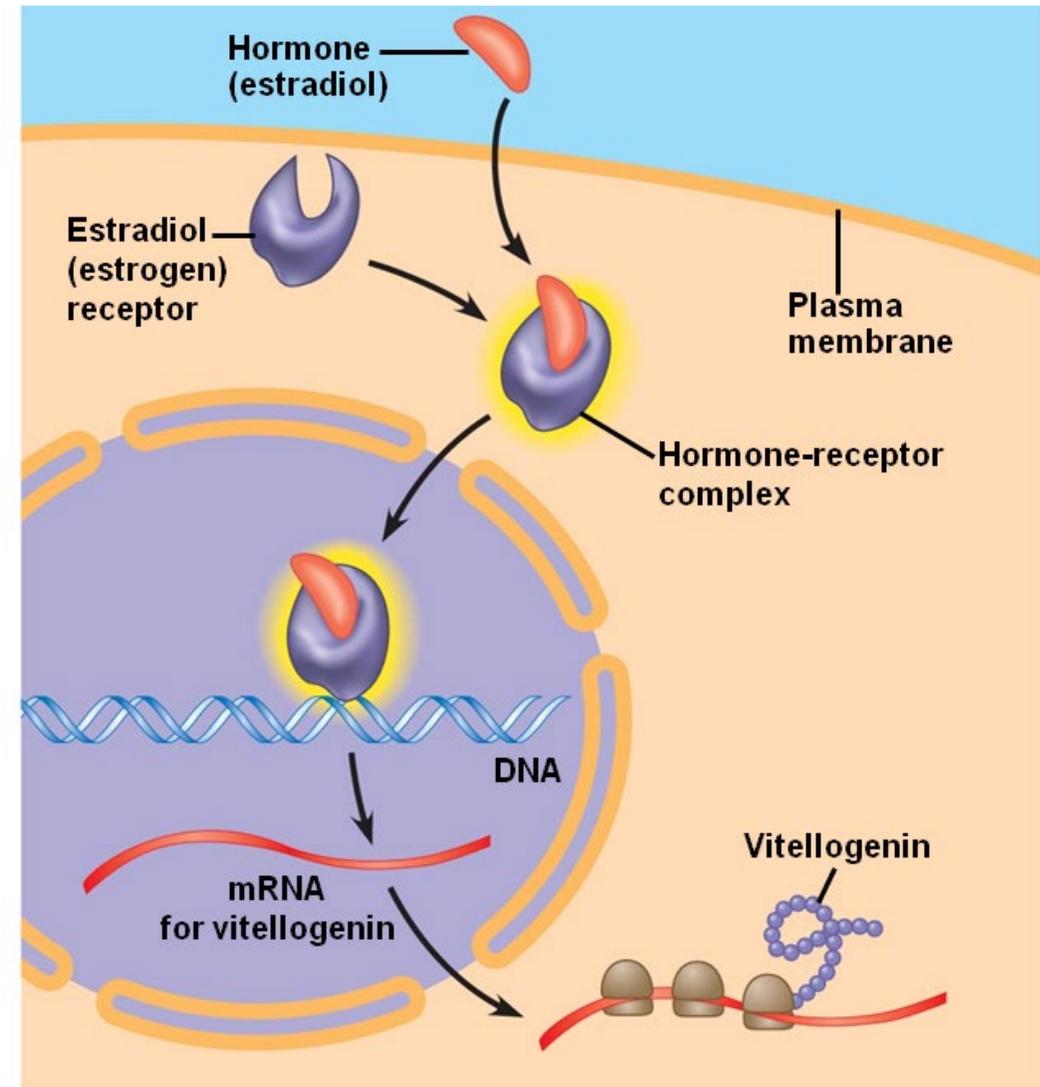


Workflow



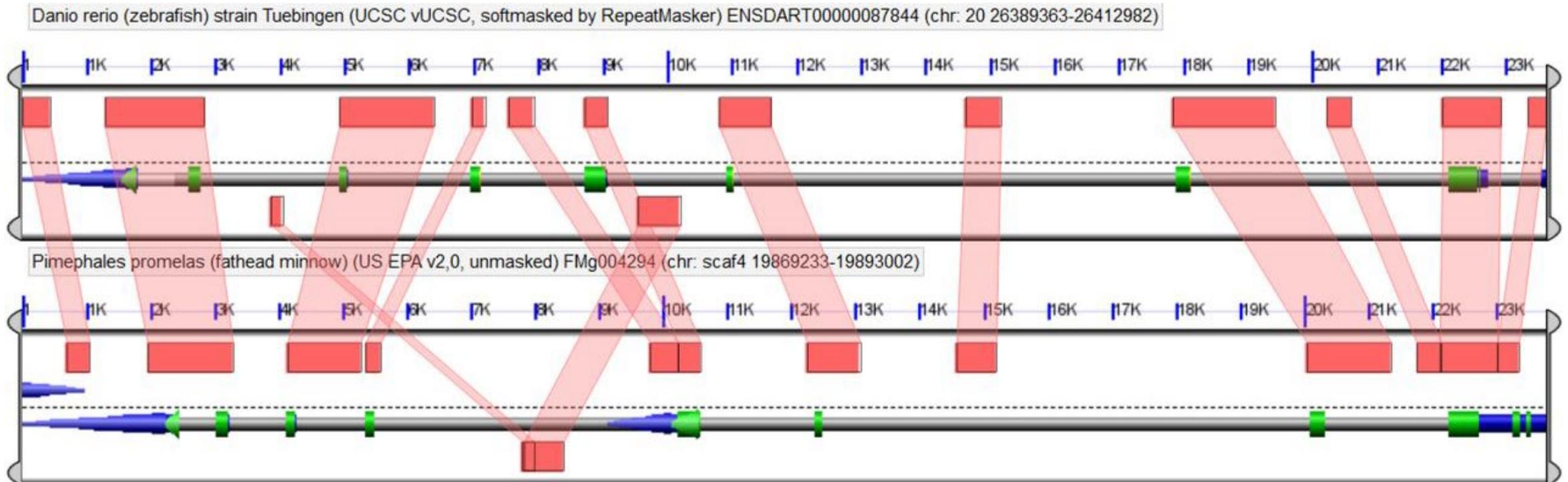
ESR1

- Estrogen Receptor- α
 - Expressed in most tissues
- Tissue specific expression
 - Protective function in brain
 - Sex differentiation
 - Reproduction
 - 7 putative isoforms in FHM
- Dysregulation implicated in disease
 - Cancer
 - Neurological disorders (Alzheimer's)
 - Coronary artery disease



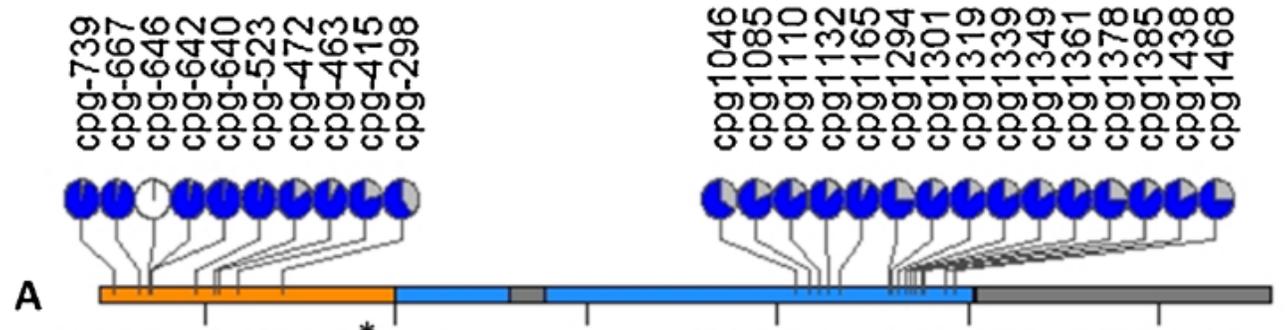
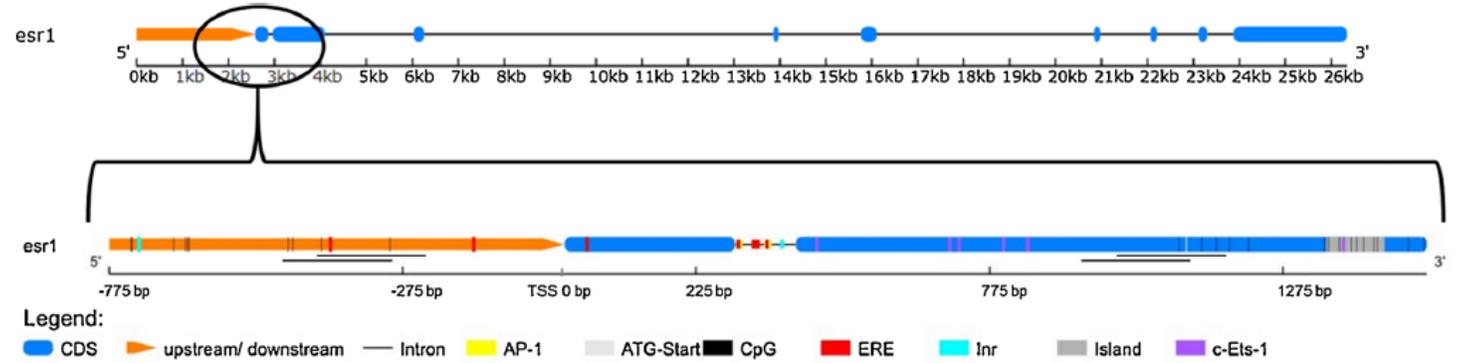
FHM & ZF ESR1

- Single copy
- Differ in isoform number
- Conserved exon order
- Intronic regions differ





FHM ESR1 Gene Structure



Differential Methylation

			Upstream of transcriptional start site									
CpG site:			-739	-667	-646	-642	-640	-523	-472	-463	-415	-258
Liver	2 Day	Low	0.96	0.96	N/A	0.96	0.96	0.96	0.96	0.96	0.005	0.96
		High	0.479	0.907	N/A	0.479	0.64	N/A	0.479	0.04	0	0.108
		♀	0.966	0.943	N/A	0.496	0.556	0.987	0.45	0	0.005	0.031
	7 Day	Low	0.895	0.691	0.689	0.691	0.689	0.689	0.141	0.689	0.689	0.94
		High	0.96	0.96	0.96	0.96	1	0.96	0.96	0.342	0.96	
	14 Day	Low	0.91	0.67	0.67	0.003	0.67	0.975	0.67	0.975	0.67	0.67
High		0.984	0.846	0.984	0.95	0.947	0.573	0.573	0.568	0.926	0.926	

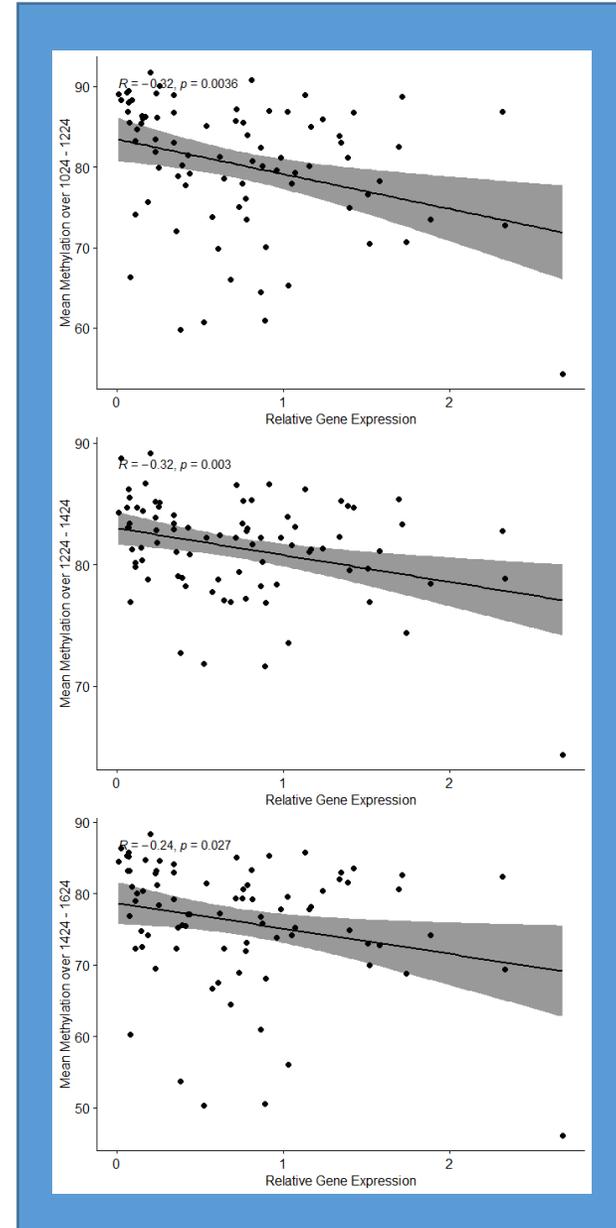
Within the coding region (first 2 exons)																
1046	1085	1110	1132	1165	1294	1301	1319	1339	1349	1361	1378	1385	1438	1468		
0.96	0.96	0.96	0.96	0.96	0.96	0.96	0.96	0.96	0.96	0.96	0.96	0.96	0.96	0.96	0.96	
0.733	0.64	0.907	0.415	0.478	0.475	0.488	0.484	0.468	0.445	0.263	0.402	0.402	0.476	0.455		
0.001	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
0.689	0.689	0.689	0.622	0.689	0.689	0.689	0.689	0.689	0.689	0.689	0.689	0.689	0.689	0.689		
0.96	0.96	0.96	0.96	0.342	0.96	0.96	0.96	0.96	0.96	0.996	0.96	0.96	0.96	0.96		
0.975	0.757	0.68	0.68	0.67	0.678	0.735	0.68	0.678	0.67	0.67	0.67	0.757	0.68	0.678		
0.573	0.769	0.846	0.846	0.846	0.984	0.926	0.947	0.947	0.984	0.95	0.984	0.943	0.984	0.984		

Brain	2 Day	Low	0.544	0.408	0.624	0.507	0.228	N/A	0	N/A	N/A	N/A
		High	N/A	0.898	N/A	0.898	0.987	N/A	0.101	0.898	N/A	N/A
		♀	0.852	0.943	0.852	0.852	0.852	N/A	0.852	0.852	N/A	N/A
	7 Day	Low	0.767	0.463	0.803	0.962	0.962	N/A	0.405	N/A	N/A	N/A
		High	0.15	0.228	0.681	0.683	0.985	N/A	0.251	0.493	0.552	N/A
	14 Day	Low	0.504	0.783	0.603	0.648	0.648	N/A	0.739	0.504	N/A	N/A
High		0.714	0.862	0.507	0.581	0.507	0.714	0.019	0.171	N/A	N/A	

0.004	0.035	0.037	0.044	0.075	0.044	0.408	0.075	0.061	0.051	0.797	0.037	0.044	0.624	0.037
0.761	0.987	0.987	0.898	0.898	0.987	0.309	0.898	0.898	0.898	0.634	0.761	0.987	0.634	0.898
0.547	0.852	0.852	0.852	0.943	0.852	0.852	0.852	0.852	0.852	0.852	0.852	0.852	0.943	0.547
0.962	0.925	0.564	0.45	0.75	0.405	0.431	0.305	0.305	0.305	0.405	0.395	0.395	0.405	0.925
0.683	0.185	0.166	0.095	0.116	0.095	0.095	0.095	0.095	0.101	0.095	0.095	0.187	0.051	0.683
0.739	0.504	0.516	0.504	0.504	0.603	0.504	0.504	0.504	0.504	0.504	0.504	0.55	0.504	0.648
0.55	0.507	0.507	0.507	0.507	0.507	0.998	0.507	0.507	0.507	0.687	0.519	0.507	0.507	0.68

ESR1 methylation and expression

- ESR1 differentially expressed in liver
 - Low and high doses
- Methylation
 - Tiled window analysis
 - Inverse correlation
 - Both concentrations
 - Near two putative ERE
 - Potential for alternative upstream promoter
 - Differences b/w ZF and FHM



Summary

- Methylation
 - Differences among tissues
 - Promoter
 - Dose-dependent methylation response - Liver
 - Female-“like” methylation pattern in promoter of males
 - Negatively correlated with Tx
 - Gene body
 - Large regional shifts in methylation in low dose males
 - Differed from female pattern
 - Potential for isoform usage
 - Lasting methylation differences

EE2 – whole genome methylation

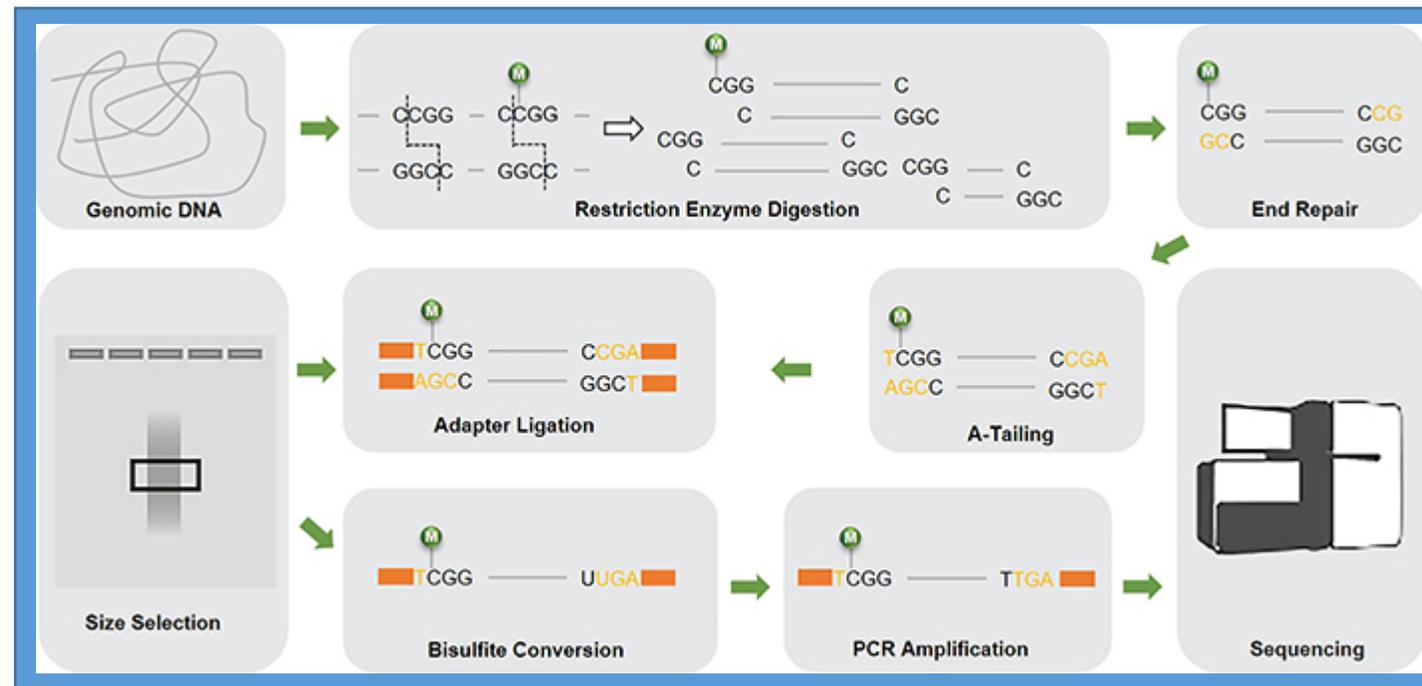


Research Questions/Drivers

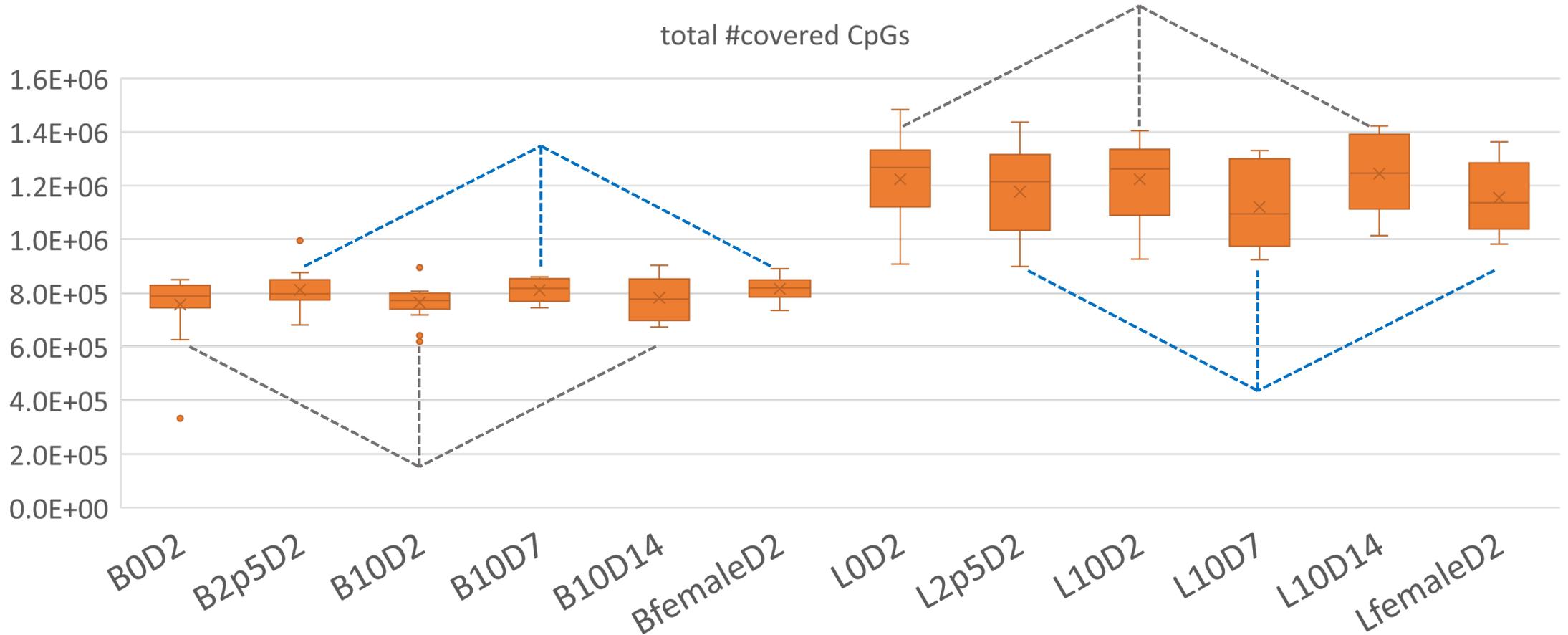
- **Biological**
 - What are main epigenetic changes associated with the short-term EE2 exposure in brain and liver.
 - Dose-specific responses
 - Kinetics of methylation
- **Methodological**
 - Limitations & benefits of reduced representation bisulfite sequencing
 - Functional quality assessment of the FHM genome assembly

Reduced Representation Bisulfite Sequencing

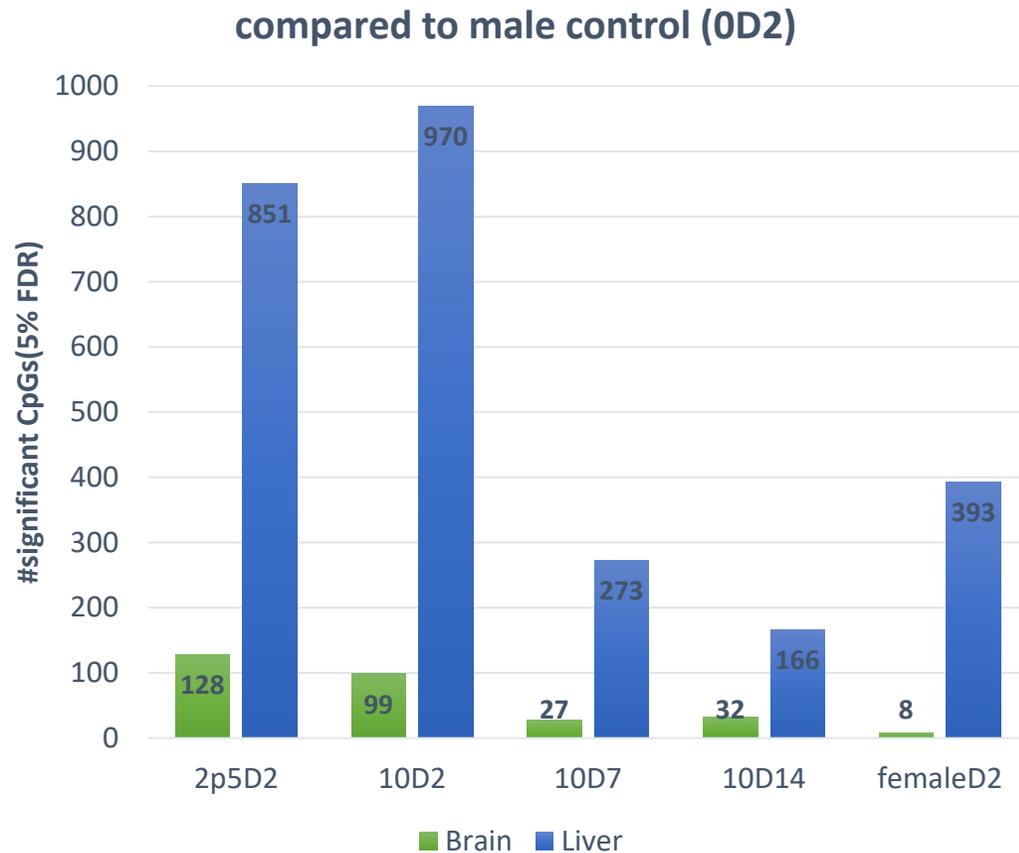
- Targets CpG enriched regions
- Single base pair resolution
- Relatively inexpensive (compared to whole genome) ~8% of total CpG
- 85% CpG islands, 60% of promoters



Total number of covered CpGs by RRBS

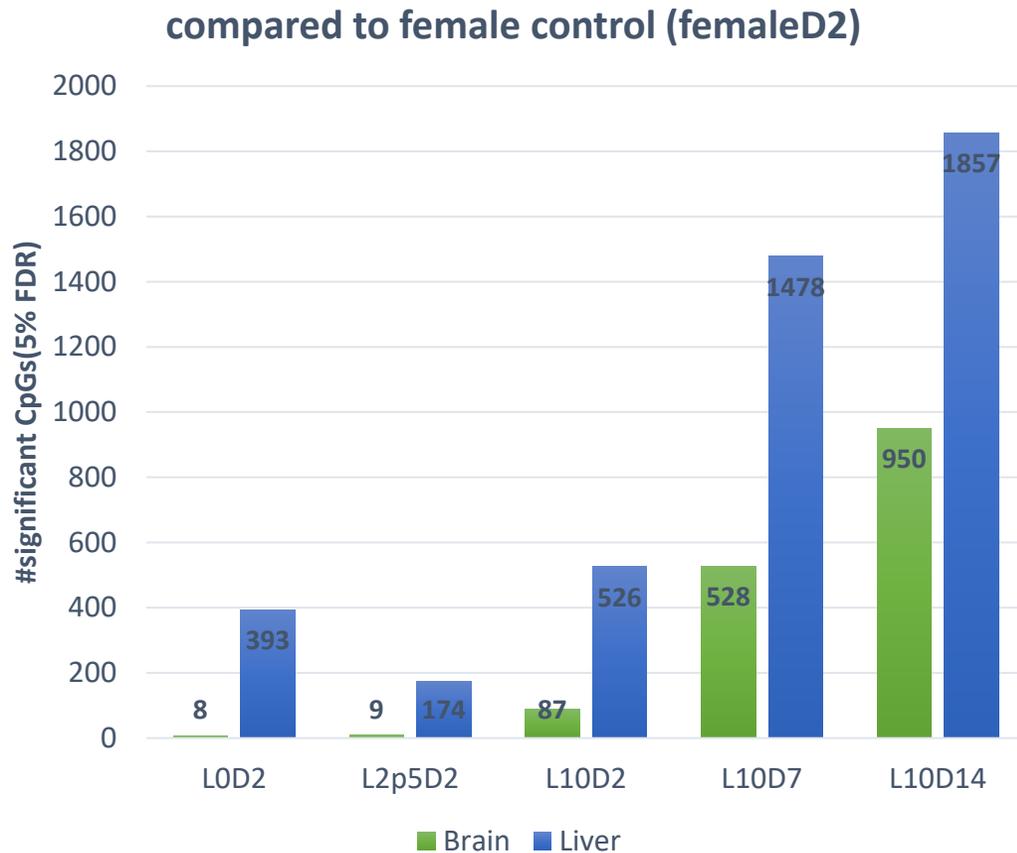


#DMCs between the male control and the other groups



- In brain, the low-dose group has the highest #DMCs, while the female control has the fewest
- In liver, the high-dose has the largest #DMCs, while the 14-day depuration has the lowest #DMCs
- In the female control, the brain tissue has much lower #DMCs than the liver tissue

#DMCs between the female control and the other groups

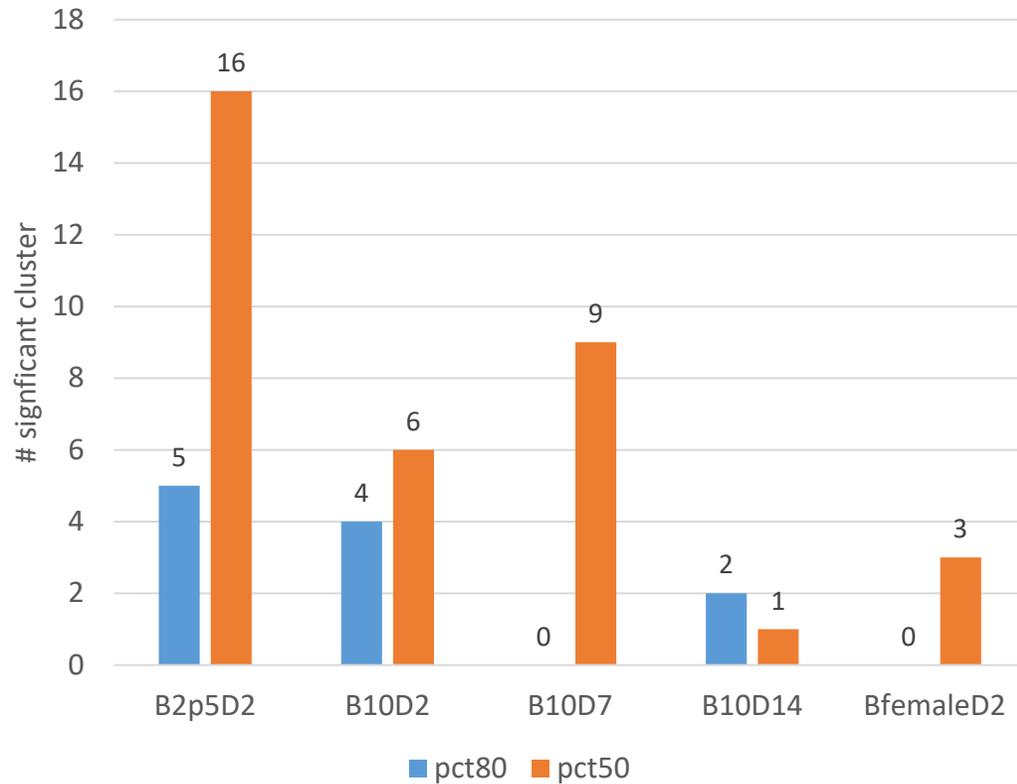


- The low-does group has the fewest #DMCs, indicating low-dose treatment might lead to male more like female in liver
- In both brain and liver, all high-dose treated groups have more much DMCs than the male control, and #DMCs is the largest in the 14-day depuration group, suggesting potentially long-lasting detrimental effects

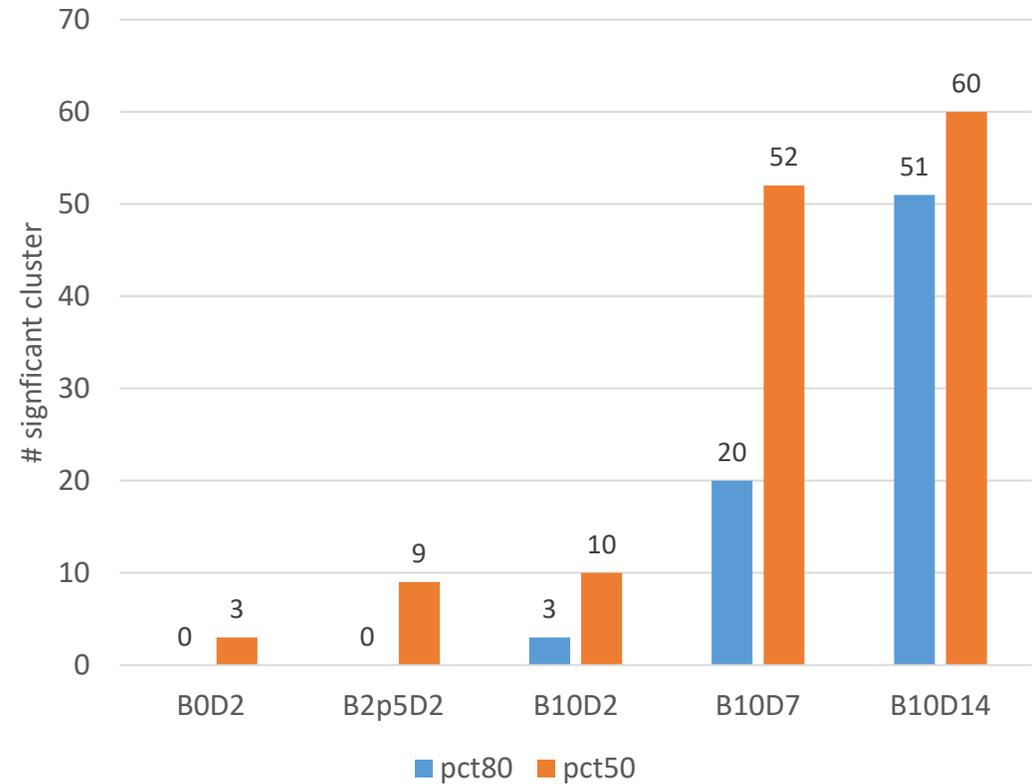
group1	group2	n_all	%promoter	%exon	%intron	%other	FDR10	FDR5	FDR1	%promFDR5	%exonFDR5	%intronFDR5	%otherFDR5
B0D2	B2p5D2	160501	2.70%	39.71%	33.11%	24.47%	242	128	29	7.8%	25.0%	32.0%	35.2%
B0D2	B10D2	147657	2.67%	39.61%	33.11%	24.62%	172	99	17	4.0%	22.2%	29.3%	44.4%
B0D2	B10D7	38912	2.37%	52.42%	25.86%	19.35%	42	27	0	0.0%	14.8%	14.8%	70.4%
B0D2	B10D14	37706	2.37%	51.88%	26.11%	19.65%	39	32	18	0.0%	18.8%	46.9%	34.4%
B0D2	BfemaleD2	39057	2.41%	52.19%	25.92%	19.48%	24	8	2	0.0%	37.5%	62.5%	0.0%
B2p5D2	BfemaleD2	50954	2.48%	52.30%	25.87%	19.35%	32	9	0	33.3%	66.7%	0.0%	0.0%
B10D2	BfemaleD2	82481	2.30%	49.40%	27.57%	20.73%	118	87	18	0.0%	37.9%	20.7%	41.4%
B10D7	BfemaleD2	244735	2.54%	39.19%	33.61%	24.66%	1098	528	178	3.6%	40.7%	28.8%	26.9%
B10D14	BfemaleD2	234376	2.58%	38.81%	33.83%	24.78%	1649	950	395	2.0%	37.9%	30.5%	29.6%
L0D2	L2p5D2	438902	2.59%	38.99%	34.19%	24.23%	1785	851	155	2.2%	33.0%	39.2%	25.5%
L0D2	L10D2	456330	2.57%	38.76%	34.33%	24.34%	1901	970	193	1.6%	41.9%	37.0%	19.5%
L0D2	L10D7	114259	2.47%	48.52%	27.83%	21.17%	605	273	69	1.5%	41.0%	30.4%	27.1%
L0D2	L10D14	129909	2.53%	47.39%	28.59%	21.49%	347	166	81	0.0%	44.6%	29.5%	25.9%
L0D2	LfemaleD2	138943	2.48%	46.96%	28.90%	21.66%	673	393	80	1.8%	45.0%	31.8%	21.4%
L2p5D2	LfemaleD2	113842	2.34%	50.46%	27.03%	20.17%	340	174	37	2.9%	29.3%	44.3%	23.6%
L10D2	LfemaleD2	139576	2.42%	46.85%	29.02%	21.70%	855	526	160	2.1%	39.9%	31.4%	26.6%
L10D7	LfemaleD2	326027	2.64%	39.42%	33.63%	24.31%	3336	1478	364	3.2%	38.7%	34.2%	24.0%
L10D14	LfemaleD2	438655	2.62%	37.50%	34.92%	24.97%	3756	1857	527	1.9%	34.7%	39.8%	23.5%

#DMRs in the brain tissue between the male control and the other groups

compared to male control (0D2)

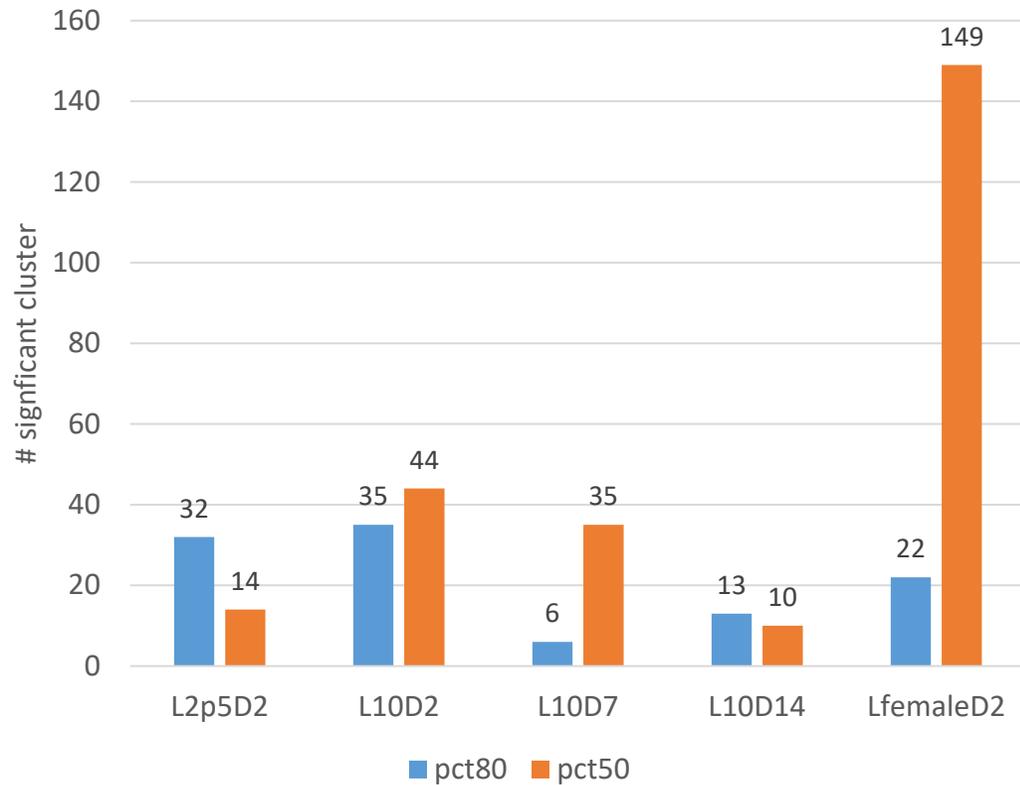


compared to female control (femaleD2)

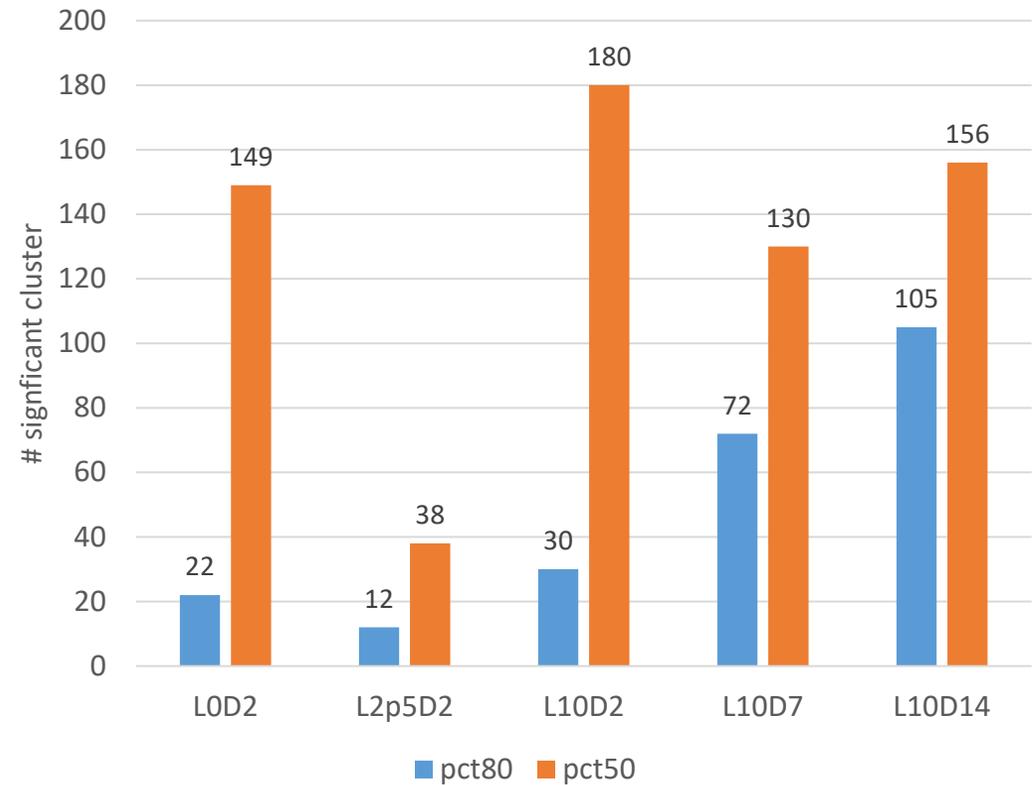


#DMRs in the liver tissue between the male control and the other groups

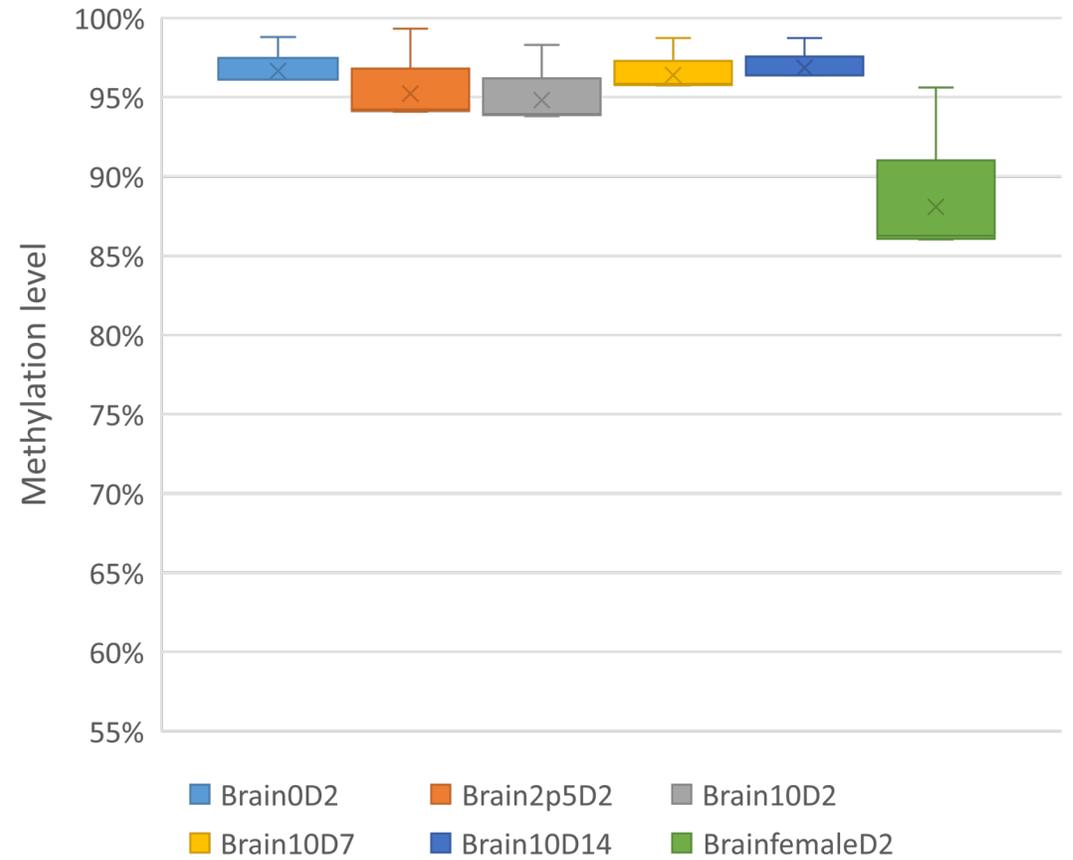
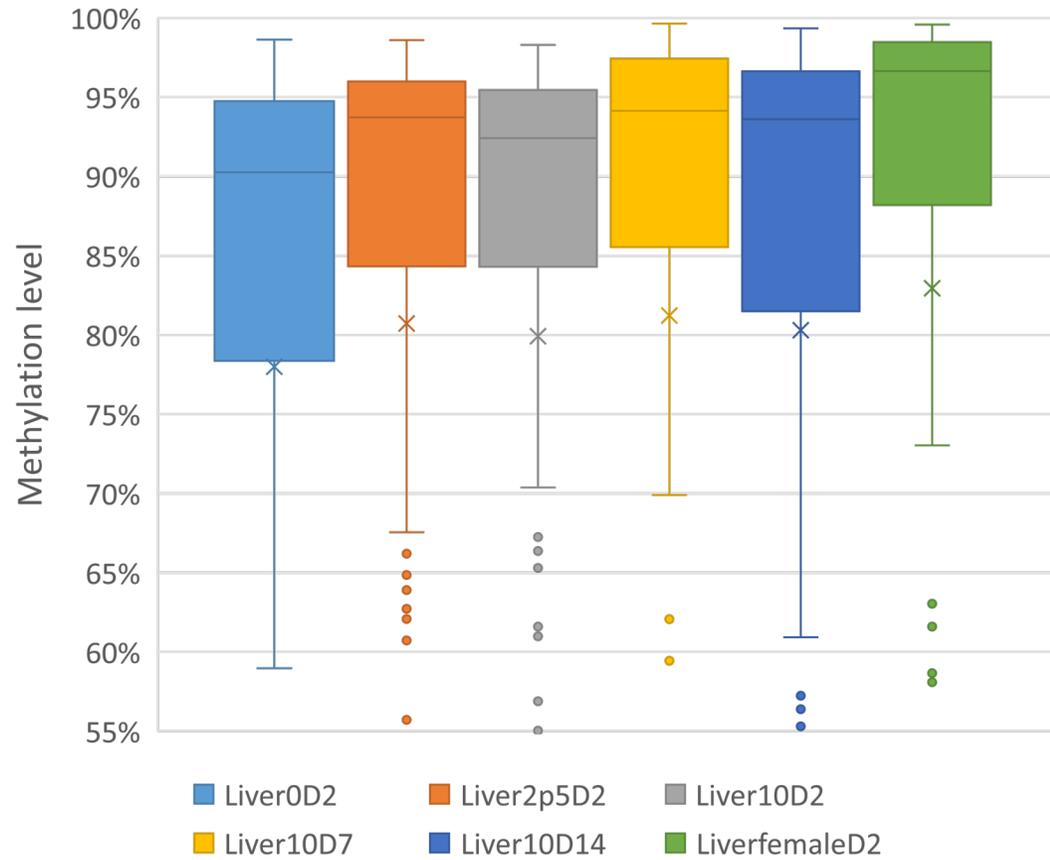
compared to male control (0D2)



compared to female control (femaleD2)



OD2-FD2: overall change patterns



GO	NS	name	Liver0D2	Liver10D2	Liver10D7	Liver10D14
GO:0000902	BP	cell morphogenesis	X	X	X	X
GO:0009653	BP	anatomical structure morphogenesis	X	X	X	X
GO:0032501	BP	multicellular organismal process	X	X	X	X
GO:0022610	BP	biological adhesion	X	X	X	X
GO:0032989	BP	cellular component morphogenesis	X	X	X	X
GO:0007155	BP	cell adhesion	X	X	X	X
GO:0048870	BP	cell motility	X		X	X
GO:0040011	BP	locomotion	X		X	X
GO:0007267	BP	cell-cell signaling	X		X	X
GO:0023052	BP	signaling	X		X	X
GO:0007275	BP	multicellular organism development	X		X	X
GO:0009790	BP	embryo development	X		X	X
GO:0030154	BP	cell differentiation	X		X	X
GO:0032502	BP	developmental process	X		X	X
GO:0048869	BP	cellular developmental process	X		X	X
GO:0007154	BP	cell communication	X		X	X
GO:0006928	BP	movement of cell or subcellular component	X		X	X
GO:0048646	BP	anatomical structure formation involved in morphogenesis	X		X	X
GO:0005886	CC	plasma membrane	X			X
GO:0016020	CC	membrane	X			X
GO:0000003	BP	reproduction			X	
GO:0050877	BP	nervous system process		X	X	X
GO:0040007	BP	growth		X	X	X
GO:0050789	BP	regulation of biological process			X	X
GO:0071840	BP	cellular component organization or biogenesis			X	X
GO:0050794	BP	regulation of cellular process			X	X
GO:0009987	BP	cellular process			X	X
GO:0065007	BP	biological regulation			X	X
GO:0016043	BP	cellular component organization			X	X
GO:0007165	BP	signal transduction			X	X
GO:0003008	BP	system process			X	X
GO:0031012	CC	extracellular matrix				X

SUMMARY

- Dose-dependent methylation differences
- Sex differences in methylation
 - Female – lower methylation in brain & slightly higher in liver
- General loss of methylation
 - Some lasting effects – up to 14 d
 - Potential for altered phenotype/adverse effects
- Low dose males similar to females based on gene expression
- Distribution of DMRs appears non-random
 - 7 d depuration – intronic -> isoform usage – (future studies)

Conclusions

- EE2
 - Targets multiple epigenetic mechanisms
 - Lasting effects
 - Potential for biomarkers of life history/exposome
 - Potential for impacts on risk estimates
 - Multiple exposures etc.
- Genome
 - Demonstrated sufficient contiguity and completeness
- Methods
 - RRBS – reasonable approach to identify methylation differences

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