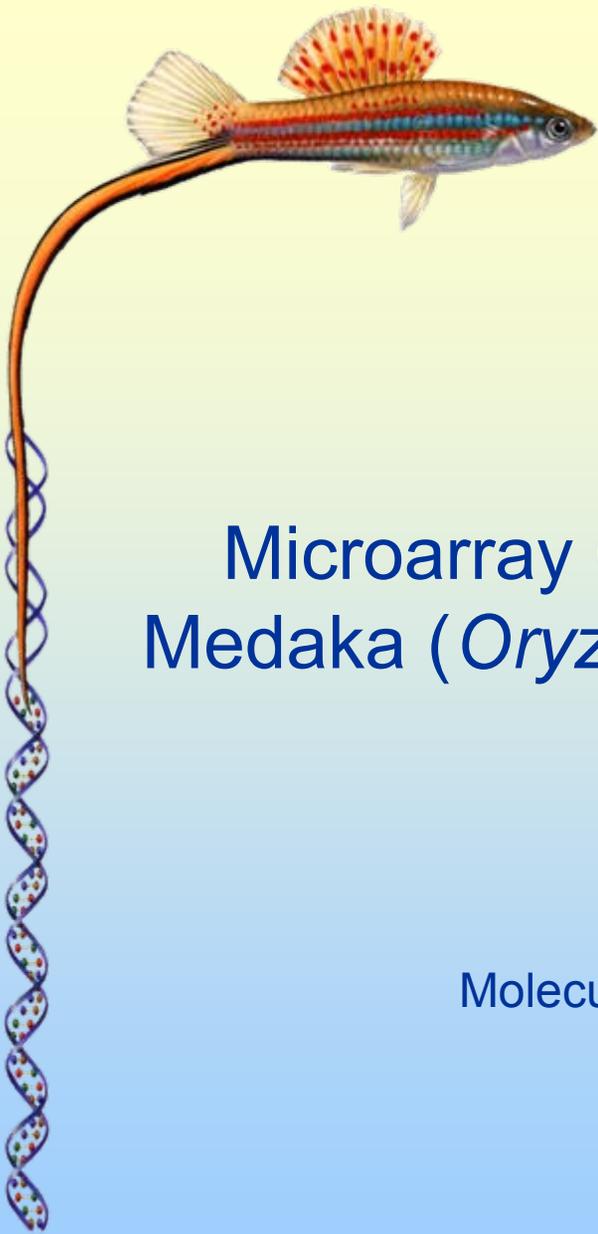




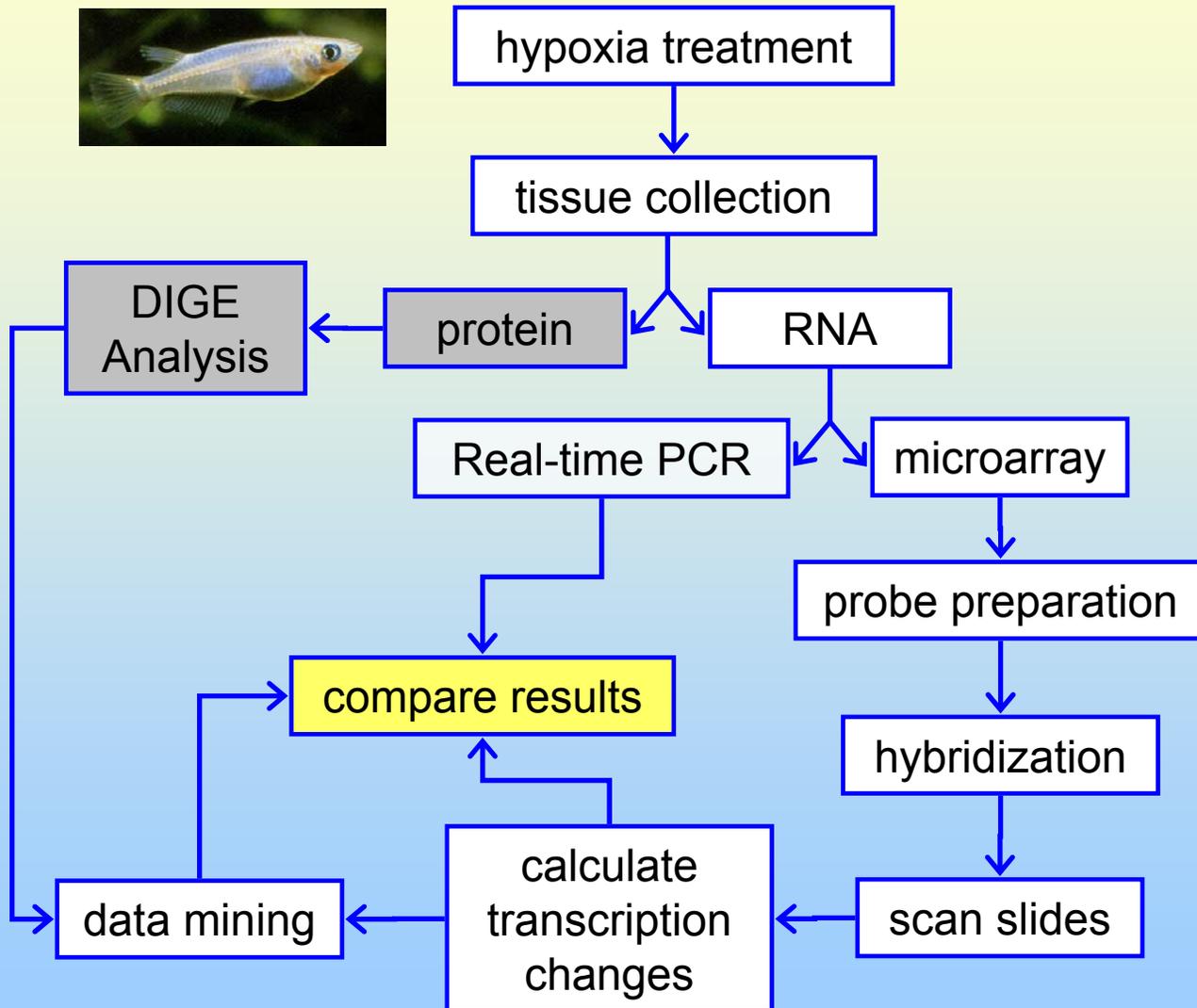
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Microarray Gene Expression Analyses in Medaka (*Oryzias latipes*) Exposed to Hypoxia

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Overview of Experimental Plan



The Medaka Model

- Short generation times of 2-4 months.
- Genome size that is smaller than other models such as zebrafish and mammals.
- A large number of medaka ESTs generated through the Medaka Genome Initiative in Japan are publicly available (http://mbase.bioweb.ne.jp/~dclust/medaka_top.html).
- Collaborative efforts with the University of Southern Mississippi

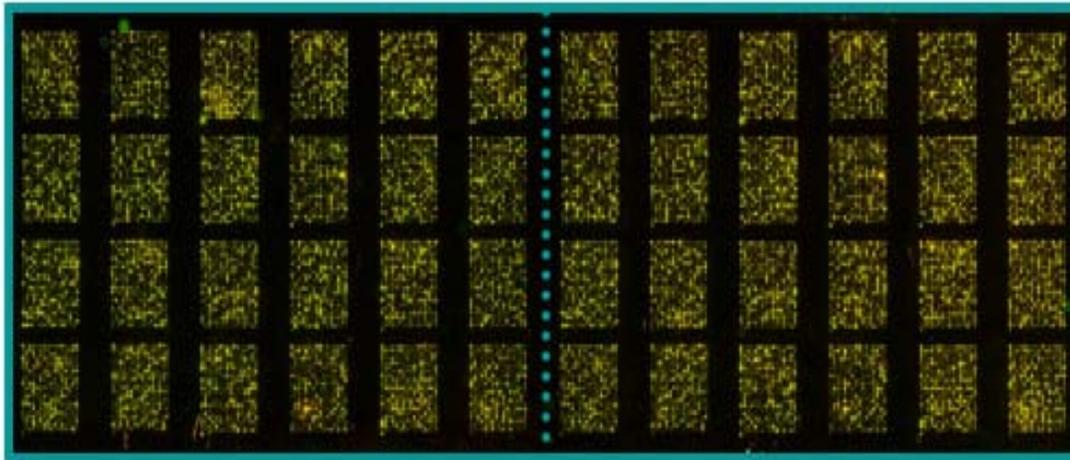


Medaka Oligonucleotide Microarray

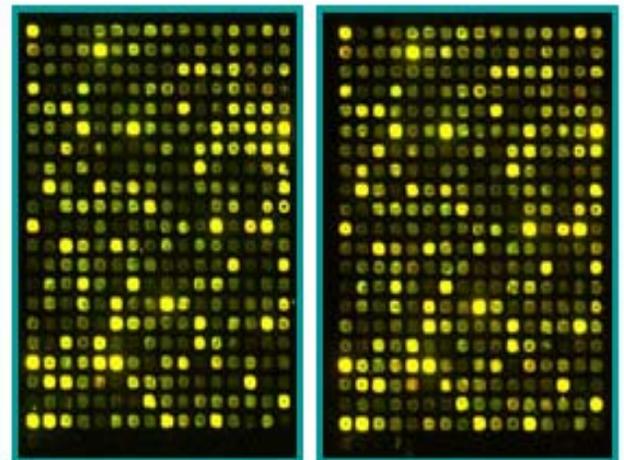
- 60-mer oligonucleotide spotted on aminosilane-coated slides.
 - $T_m=75\pm 5^\circ\text{C}$.
 - Sequences within 1kb of 3' end of the coding region.
 - Maximum length of simple repeats: 6 bases.
 - BLAST searched to verify oligo specificity.
 - yield: 8,046 ESTs meet criteria.
- (+) control is medaka cytoplasmic β -Actin.
- (-) control is a yeast tRNA.
- Oligos synthesized by Integrated DNA Technologies in 384-well plates.
 - Quality control ran on every oligo ensuring full-length oligonucleotide targets.

Medaka Oligonucleotide Microarray

- 8,046 features (e.g. 60-mer oligo targets) plus controls spotted in duplicate to verify signal consistency across each slide.
- 337-338 features per sub-block (including controls).
- Spot quality is excellent.
 - Clean and even.
 - Minimum doughnut-shaped spots.
 - Allows feature data to be measured consistently.



Duplicated meta-blocks



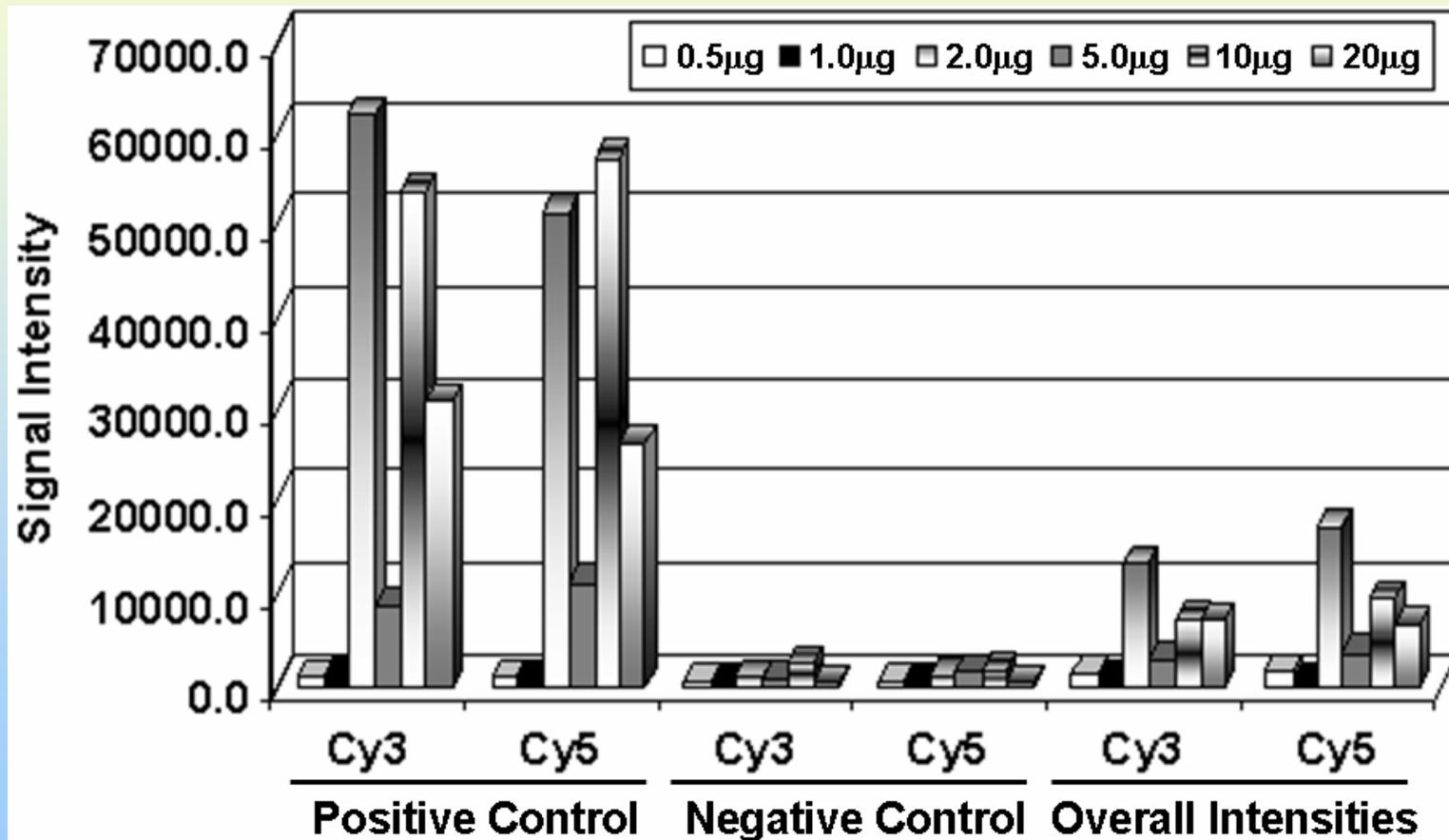
Duplicated sub-blocks

a standard size microscope slide

Gene Detection Capability Standard vs. Amplification Protocols

Protocol	Total RNA (ug)	Number of Genes Detected			
		Cy3		Cy5	
		Mean	Percent	Mean	Percent
Standard	20	7471	93%	7565	94%
	10	7578	94%	7571	94%
	5	3297	41%	2855	35%
Amplification	2	7580	94%	7580	94%
	1	7136	89%	7501	93%
	0.5	6602	82%	5717	71%

Signal Intensity Standard vs. Amplification Protocol



Treatment Method

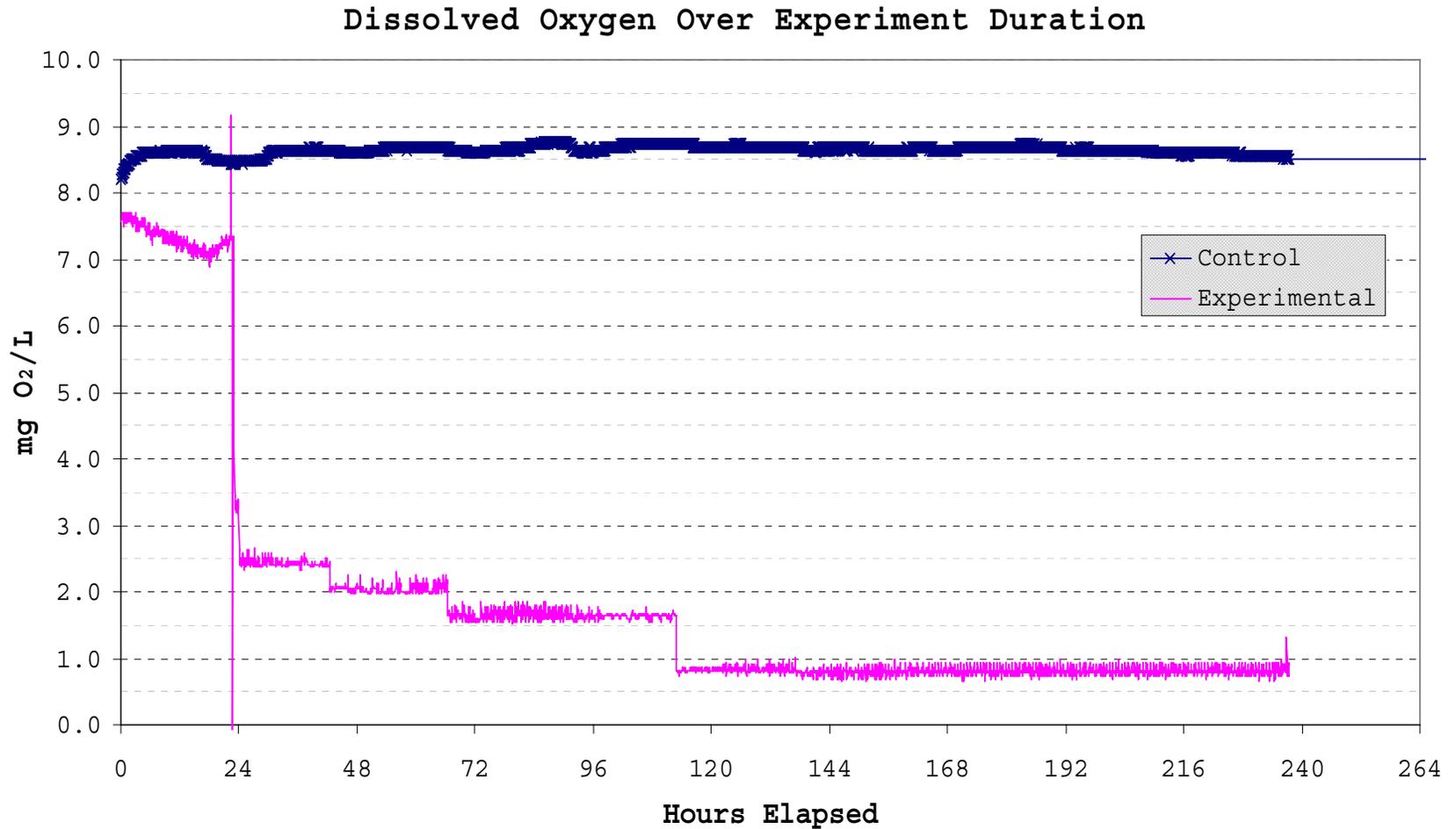
- Biospherix OxyCycler oxygen control system



Treatment Method

- Biospherix OxyCycler oxygen control system
 - Measures oxygen levels both in the water and headspace above the water.
 - precision = ± 0.1 mg O₂/L
 - Adjusts gas infusions into the system via a feedback loop to achieve desired set point.
 - Real-time data monitoring & continual logging.
 - Allows manual or automatic control including recipes.

Oxygen Levels During Treatment



Visual Medaka Responses

- In aquaria:
 - Heavy respiration
 - Lethargic
 - Lack of appetite
- Upon dissection:
 - Very dark and enlarged gall bladders – a possible indication of liver stress.

aRNA Probe Synthesis

- 2 µg total RNA template, amplification protocol.
- First strand synthesis.
 - Reverse transcriptase (Superscript III™)
 - T7-(dT)₂₄ primer
- Second strand synthesis
 - DNA Ligase, DNA Polymerase I, RNase H, T4 DNA polymerase.
 - Clean up with Qiagen Qiaquick PCR Purification column.
- *In vitro* transcription of amplified RNA (aRNA)
 - Cy-3 (fluoresces green) or Cy-5 (fluoresces red) labeled CTP.
 - T7 RNA polymerase
 - Clean up with Qiagen RNeasy Kit.

Hybridization

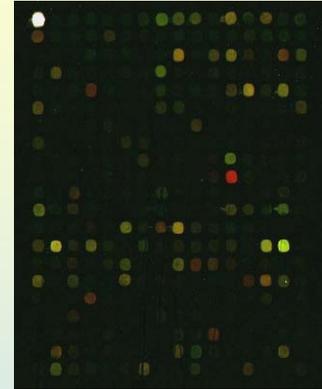
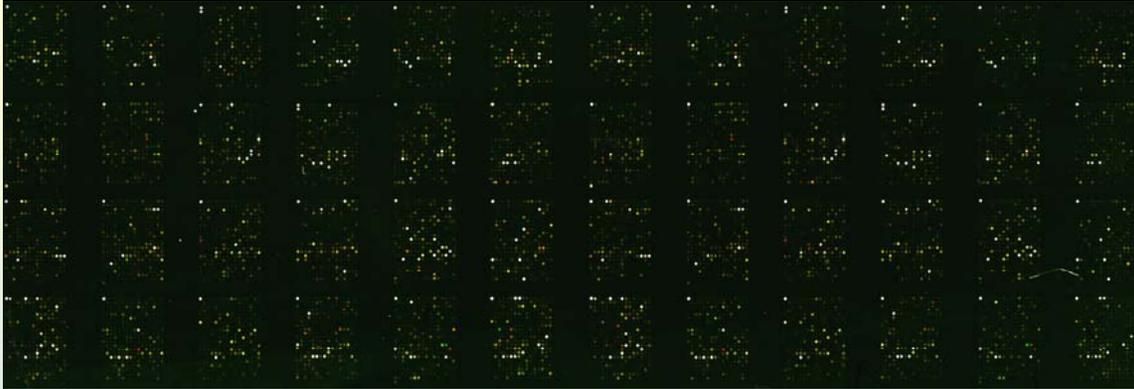
- UV crosslinking to bind oligos to slide.
- Prehybridized slides with BSA to block background noise.
- Hybridization
 - COT-1 DNA blocks highly repetitive, non-specific binding to targets.
 - Poly d(A) blocks non-specific binding to any poly-T tails.
 - yeast tRNA binds to (-) control (has no fluorescent dye).
 - Hybridization buffer, mostly formamide with SSC & SDS to aid in selecting for specific binding.
 - Hybridized overnight, 16-20 hours.
- Wash steps
 - 4 wash steps
 - Stepping down concentrations of SDS and SSC
 - Stepping down temperature
 - Spin dry



Genomic Solutions Hybstation

Analysis

- Microarrays are scanned with an Axon GenePix 4000B scanner.



- Red: Cy5 sample most abundant
- Green: Cy3 sample most abundant
- Yellow: Cy5 & Cy3 samples equally abundant
- White: Feature is saturated with dye.

- GenePix software flags “bad” data
 - irregular shapes
 - $< 70\%$ of feature pixels are at least 2 std. deviations above background.
 - Scratches, splotches, etc.

- GenePix software normalizes to the global mean.
 - Assumption that most genes' transcription levels are unchanged.

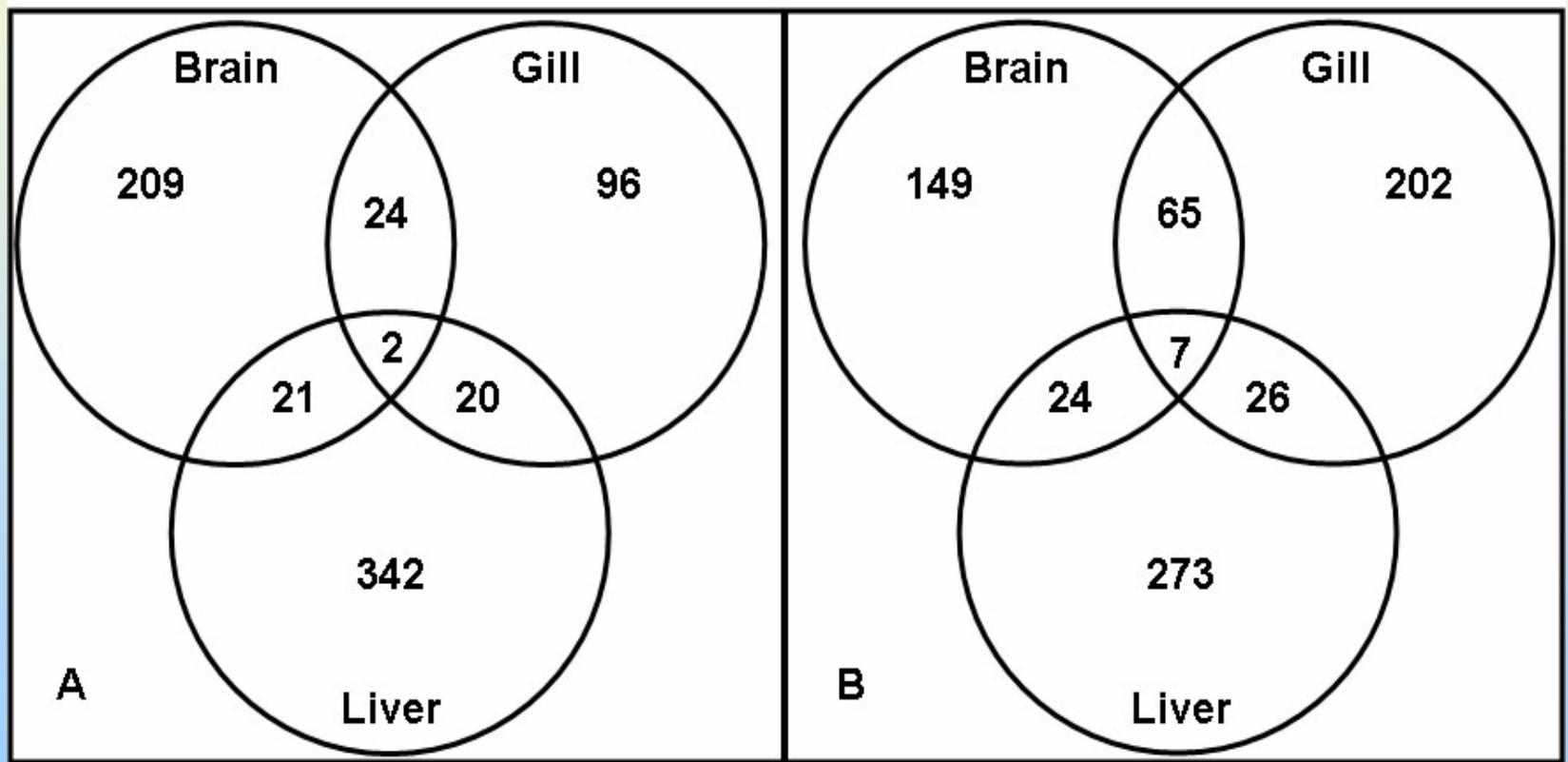
Analysis

- Repeats compared to one another as well as to their corresponding dye-flips.
 - Dye-flips counterbalance any potential biases in dye signal.
 - Recall: a minimum of 24 repeats (12 per dye-flip) are collected.
- Verify reproducibility via a Pearson correlation, $r \geq 0.85$.
- Intensity data subjected to a 1-sample t-test or SAM analysis to test for significance.
 - p -value ≤ 0.05
 - q -value = 0
- Compute fold-increases
- Choose spots for verification via real-time PCR.

Analysis

- Differentially expressed features mined for re-annotation, gene ontology, and pathway.
 - Swiss-Prot
 - Database for Annotation, Visualization, and Integrated Discovery (DAVID)
 - Kyoto Encyclopedia of Genes and Genomes (KEGG)

Hypoxia Responsive Features by Tissue



A. Transcription up-regulated in medaka
 q -value = 0

B. Transcription down-regulated in medaka

Features Exhibiting Common Expression in All 3 Tissues

<i>GenBank Acc #</i>	<i>Match_id</i>	<i>Symbol</i>	<i>Description</i>	<i>e-value</i>	<i>Bit Score</i>	<i>Functional Category</i>
Hypoxia induced genes in medaka						
BJ005346			EST			
BJ529972			EST			
Hypoxia suppressed genes in medaka						
AB009569	Q9WVL4	GRK1	RK_MOUSE(Rhodopsin kinase (RK))	0	744	Signal transducer
AB023489	P18910	NPR1	ANPRA_RAT (Atrial natriuretic peptide A-type receptor)	0	1263	Signal transducer
AB041330			Oryzias latipes c GnRH-II mRNA for preprogonadotropin-releasing hormones-II			
AU169186	Q8K097	FAIM2	FAIM2_MOUSE(Fas apoptotic inhibitory molecule 2 (Lifeguard protein))	1.00E-04	45.1	Anti-apoptosis
AV669091	P70158	SMPDL3A	AS3A_MOUSE(Acid sphingomyelinase-like phosphodiesterase 3a precursor)	3.00E-33	139	Signal transducer
D89724	Q8UW64	N/A	PSB9_ORYLA(Proteasome subunit beta type 9 precursor)	4.00E-114	409	Hydrolase
Y11252	P55260	ANXA4	ANXA4_RAT(Annexin A4 (Annexin IV))	1.00E-119	428	Binding

Gene Ontology Conclusions

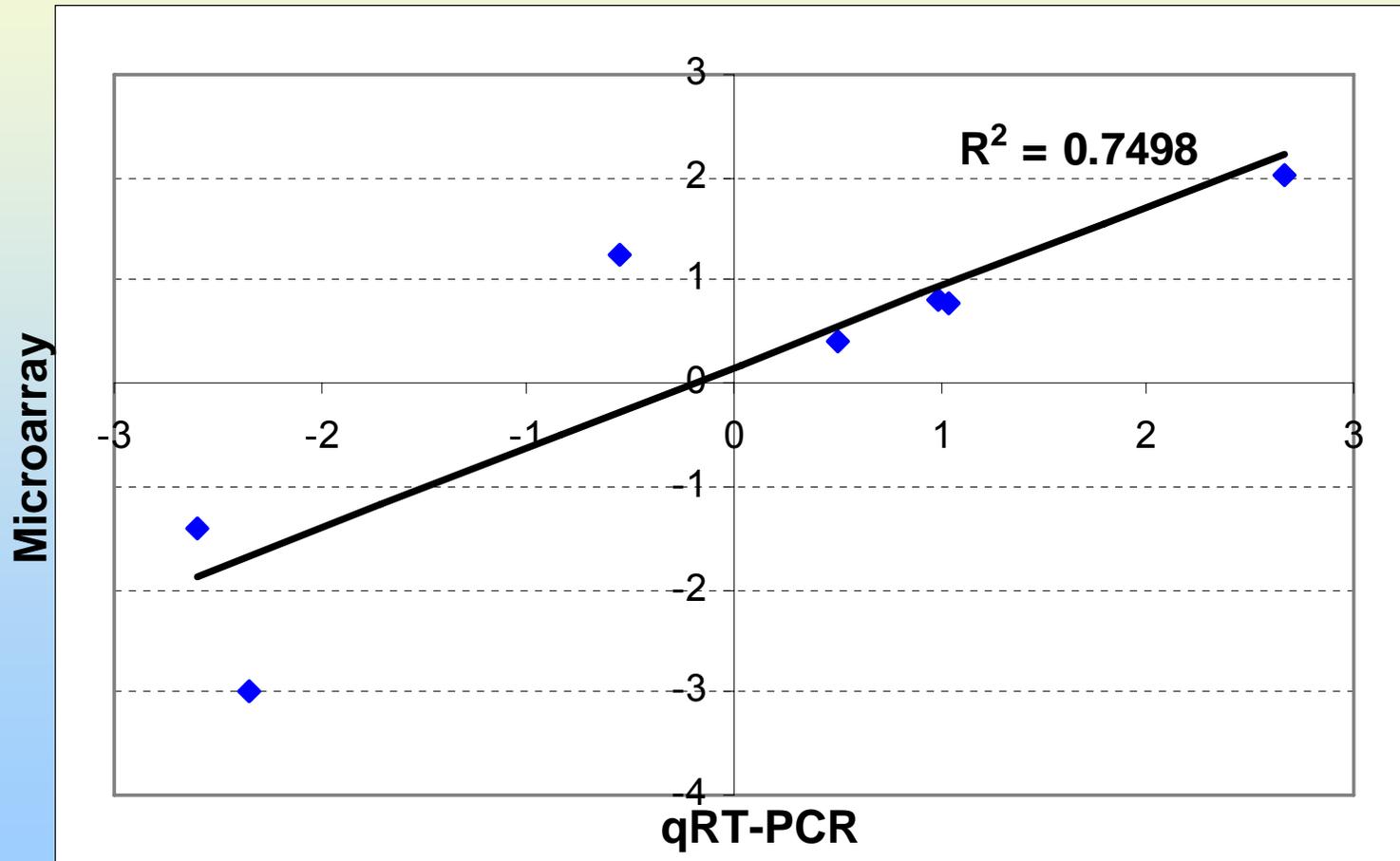
- Gene ontology data was available for 424 of the identified spots.
 - The majority fell into various metabolism ontology groups (general, protein, mRNA, etc.). Of these, most were down-regulated.
 - Also represented groups include cell maintenance, transport, and the ubiquitin-cycle, among others.
 - Overall, these results imply an energy conservation response and slow-down of general metabolic activities.
 - Recall (lethargic fish, lack of appetite)

GO Group	Brain		Gill		Liver	
	Up ^a	Down ^b	Up	Down	Up	Down
Cell Maintenance					28	
Cytoskeleton						8
Fatty Acid Metabolism						3
Intracellular Signaling Cascade	6					
Metabolism		32			57	39
mRNA Metabolism		4		3	4	5
mRNA Processing		4			4	4
Neurogenesis	4					
Neurotransmitter Transport		2				
Oxygen and Reactive Oxygen Species Metabolism						4
Protein Biosynthesis				5		7
Protein Catabolism		5				
Protein Metabolism		14			27	20
Protein Modification						10
Response to Endogenous Stimulus	3					
Response to Hormone Stimulus	2					
RNA Metabolism		5			8	5
RNA Processing		4			6	5
RNA Splicing		4			3	3
rRNA Metabolism				2		
rRNA Processing				2		
Superoxide Metabolism						2
Translation		3	2			
Transport		8			38	11
Ubiquitin Cycle					6	
Ubiquitin-Dependent Protein Catabolism						3
Total	15	85	2	12	181	129

^a number of up-regulated genes.

^b number of down-regulated genes.

Real-time PCR Validation



log2 scale

Conclusions

- Our medaka microarray yields reproducible data which correlates well with results from real-time PCR analysis, allowing for the calculation of changes in transcript regulation with high confidence levels.
- Over one thousand transcripts are found to vary their abundance with hypoxia exposure.
- Data mining offers glimpses into pathways and disorders associated with changes in environmental stimuli.
- More work is necessary to determine what transcripts are consistent over a set of experiments analyzing both RNA and protein. These will be pursued as potential biomarkers.

Future Plans

- Hypoxia exposures
 - kinetic response
 - episodic oxygen levels
- Heavy metal exposures
- Combinations of hypoxia and heavy metal exposures.

Microarray Gene Expression Analyses in Japanese Medaka (*Oryzias latipes*) Exposed to Hypoxia

Melissa Wells, Dr. Zhenlin Ju, Sheila Heater, and
Dr. Ronald Walter

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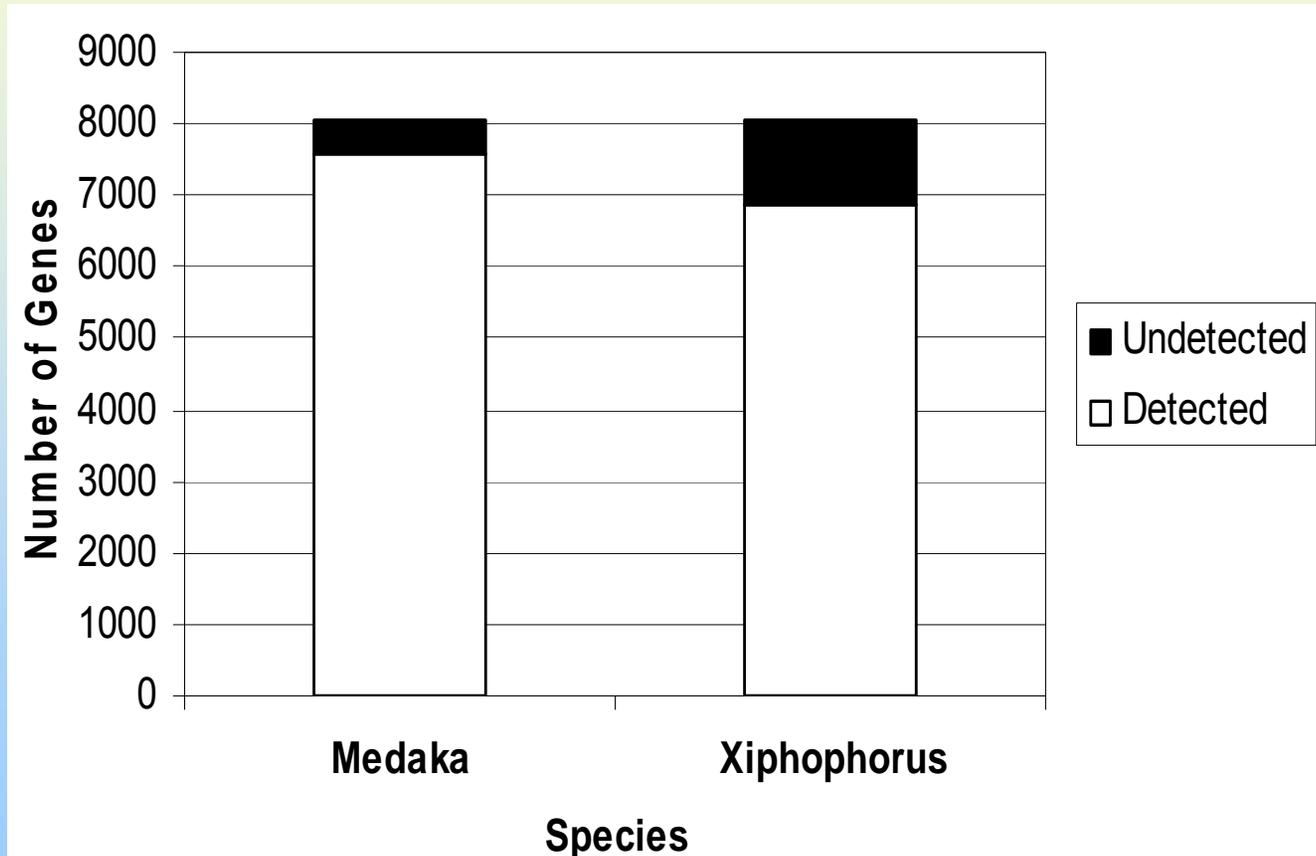
Publications

- Oehlers, L.P., Heater, S.H., J., Rains, J.D., Wells, M.C., David, W.M., and R.B. Walter (2004) Gene structure, Purification, and Characterization of DNA polymerase β from *Xiphophorus maculatus*. *Comp. Physiol. & Biochem.*, 138(3):311-324.
- David, Wendi M., David L. Mitchell, and Ronald B. Walter (2004) DNA Repair in Hybrid Fish of the Genus *Xiphophorus*. *Comparative Physiology & Biochemistry*, 138(3):300-310.
- Heater, Sheila H., Leon P. Oehlers Jr., J. Douglas Rains, and Ronald B. Walter (2004). DNA Polymerase β mRNA and Protein Expression in *Xiphophorus* fish. *Comparative Physiology & Biochemistry*, 138(3):325-334.
- Ruymgaart, Arnold, Heater, Sheila H., Oehlers Jr., Leon P., and Ronald B. Walter (2004). Gene structure and Purification a Fappy-N-Glycosylase (FEN-1) from *Xiphophorus maculatus*. *Zebrafish* 1(3): 273-285.
- Oehlers, L.P., Perez, A.N., and R.B. Walter (2005) MALDI-TOFMS of 4-sulfophenyl isothiocyanate-derivitized peptides on Anchor-Chip sample supports using sodium-tolerant matrix 2,4,6-trihydroxyacetophenone and diammonium citrate. *Rapid Comm. Mass Spectrometry*, 19(6):752-758.
- Ju Z., Wells, M. W., Kazianis, S., Rains, J. D. and R. B. Walter (2005). An *in silico* mining for simple sequence repeats from expressed sequence tags of zebrafish, medaka, *Fundulus* and *Xiphophorus*. *In Silico Biol.* 5, 0041 [pgs.1-16]
- Heater, S.J., Rains, J.D., Braden, A.R.C., Gilmer, S.M., and R.B. Walter (2006) Cloning of *JunA* and *JunB* gene homologues and mRNA expression levels in two *Xiphophorus* melanoma models. *Zebrafish*, 3(1): 11-22.
- Walter, R.B., Ju, Z., Martinez, A., Amemiya, C.T., and Samollow, P. (2006) Genomic Resources for *Xiphophorus* Research. *Zebrafish*, 3(1): 53-63.
- Ju, Z., Wells, M. C., and R. B. Walter (2006). DNA microarray technology in toxicogenomics of aquatic models: methods and applications. *Comparative Physiology & Biochemistry, Part C, in press.*
- Oehlers, L.P., Perez, A.N., and R. B. Walter (2006). Detection of hypoxia-related proteins in medaka (*Oryzias latipes*) brain tissue by difference gel electrophoresis and *de novo* sequencing of 4-sulfophenyl isothiocyanate-derivitized peptides by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, *Comparative Physiology & Biochemistry, Part C, in press.*
- Ju, Z., Wells, M.C., and R.B. Walter (2006). Multiple Tissue Gene Expression Analyses in Japanese Medaka (*Oryzias latipes*) Exposed to Hypoxia. *Comparative Physiology & Biochemistry, Part, in press.*
- Heater, S.J., Rains, J. D., Wells, M. C., Guerrero, P. A., and R. B. Walter (2006). Perturbation of DNA repair gene expression due to interspecies hybridization, *Comparative Physiology & Biochemistry, Part C, in press.*
- Walter, Ronald .B., Hazlewood, Leona, Kazianis, Steven. (2006) The *Xiphophorus* Genetic Stock Center Manual, 1st edition, Klaus D. Kallman & Manfred Schartl, Eds.,113 pages, Texas State University.
- Yang, H., Hazelwood, L., Heater, S. J., Guerrero, P. A., Walter, R.B., and T.R. Tiersch (2006). Production of F1 interspecies Hybrid Offspring with Cryopreserved Sperm from a Live-bearing Fish, the Swordtail *Xiphophorus helleri*. *Biology of Reproduction. in press.*

Sources

- <http://www.ncbi.nlm.nih.gov/>
- <http://www.mri.psu.edu/faculty/pantano/pubs/materials-today-2004.pdf>
- <http://www.yd-g.co.jp/medaka.jpg>
- http://mbase.bioweb.ne.jp/~dclust/medaka_top.html
- Bairoch, A., Boeckmann, B., 1994. The SWISS-PROT protein sequence data bank: current status. *Nucleic Acids Res* 22, 3578-3580.
- Dennis, G., Jr., Sherman, B.T., Hosack, D.A., Yang, J., Gao, W., Lane, H.C., Lempicki, R.A., 2003. DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol* 4, P3.
- Ju, Z., Wells, M.C., and Walter, R.B. DNA Microarray technology in toxicogenomics of aquatic models: Methods an applications. *Comparative Biochemistry and Physiology, Part C*. 2006. *in press*.
- Ju, Z., Wells, M.C., Heater, S.J., and Walter, R.B. Multiple tissue gene expression analyses in Japanese medaka (*Oryzias latipes*) exposed to hypoxia. *Comparative Biochemistry and Physiology Part C*. 2006. *In press*.
- Kanehisa, M., Goto, S., 2000 KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Res* 28, 27-30.
- Papandreou, I., Powell, A., Lim, A.L., Denko, N., 2005. Cellular reaction to hypoxia: sensing and responding to an adverse environment. *Mutat Res* 569, 87-100.
- Wakamatsu, Y., Pristysznyuk, S., Kinoshita, M., Tanaka, M., Ozato, K., 2001. The see-through medaka: a fish model that is transparent throughout life. *Proc Natl Acad Sci U S A* 98, 10046-10050.

Our Medaka Array Successfully Detects *Xiphophorus* Genes



Update – Current Processing & Techniques

- Employing an indirect labeling protocol of our aRNA probe.
 - Amino-allyl group acts as spacer, reducing crowding to facilitate dye-binding.
- Step-down hypoxia treatments
 - Allows time for the fish to adjust to changing conditions.
 - Improve survival rates over time imply physiological adaptations.

Sample Genes with Regulation Changes

Down-regulated genes involved in the proteasome pathway in medaka brain.

GenBank No.	Description	Fold Change (Log ₂)	
		Mean	Std
BJ743818	Proteasome 26S subunit ATPase 1 (<i>RPT2</i>)	-1.9	0.19
D89724	Proteasome subunit beta type 9 precursor (<i>P9beta</i>)	-2.0	0.49
BJ716044	26S proteasome regulatory subunit (<i>RPN8</i>)	-1.9	0.40
BJ028102	Proteasome activator 28-beta subunit (<i>PA28beta</i>)	-1.2	0.16

Sample Genes with Regulation Changes and Associated Diseases

