Supplementary Material for "Methylmercury-induced changes in gene transcription associated with neuroendocrine disruption in largemouth bass (*Micropterus salmoides*)"

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Validation of largemouth bass microarray

The largemouth bass microarray used for the gene expression studies has been validated in several previous studies, and provides good agreement among gene expression measurements made by the array and by qPCR. Garcia-Reyero et al. (2008) examined expression of several genes in liver and gonad by both qPCR and the LMB microarray. Two highly upregulated genes were detected by both qPCR and microarray, but the degree of induction detected by the microarray was less than that detected by qPCR. Importantly, 3 genes that did not show significant changes in expression when measured by qPCR also did not show significant changes in expression by microarray analysis. Among 5 moderately down-regulated genes detected by qPCR, only one showed significant down-regulation by microarray analysis. In a separate study of gene expression changes in hypothalamus, the LMB microarray results were in agreement

with qPCR results for 4 out of 5 up-regulated genes (Martyniuk et al., 2010). However, 1 up-regulated gene and 1 down-regulated gene identified by qPCR gave opposite results when measured on the LMB microarray. The down-regulated gene also gave opposite results for expression measured by qPCR and microarray in an independent experiment, suggesting that for this gene, the qPCR primers and the microarray probe are not targeting the same transcript. In a study of gene expression changes in largemouth bass liver, two up-regulated genes and two unresponsive genes were validated by qPCR (Martyniuk et al., 2011). Finally, two seasonally up-regulated genes in LMB ovary were validated by qPCR with results similar to the microarray (Martyniuk et al., 2013). Overall, the LMB microarray has good agreement with qPCR measurements of gene expression, and may be more conservative than qPCR analysis.

Additional Details on LMB and the Health Index

External lesions were limited at the St. Marys, Santa Fe, and Wekiva field sites. However, significant differences were found (á=0.05) between fish from Wekiva and Santa Fe. Wekiva was found to have the lowest proportion of lesions at 0.06, followed by St. Marys with 0.10, and Santa Fe with 0.18. Light fin fraying, while seen at all sites, was not recorded as a lesion. Moderate fin fraying was recorded as being the dominant fin lesion at Santa Fe (40%). Body lesions, generally associated with parasites, were found on two fish from both Santa Fe and St. Marys. Two additional parasitic lesions were found on the head region of Santa Fe fish. Parasites were found in the gills of fish from all three sites with Wekiva having the highest occurrence (32%). Additional gross gill lesions included a broken gill arch from both Wekiva, and Santa Fe, and a completely eroded gill filament noted in a St. Marys fish.

Histological analysis of livers showed that parasites were prevalent at all three sites. Wekiva fish had the highest degree of infestation. Other liver pathologies, which occurred only in Wekiva fish, included minimal cytoplasmic degeneration (12%) and minimal focal necrosis (12%). Granulomas were found at all three sites with Santa Fe have the highest incidence (21%). Mild bile duct proliferation was found in one fish from Santa Fe, and minimal arterial lesions occurred in one St. Marys fish.

Similar to liver, parasites were the dominant pathology found in the spleen, and fish from Wekiva had the highest severity ratings of parasitic infestation. Incidences and severity levels for parasites were much lower in St. Marys and Santa Fe fish. Other pathologies were uncommon, and included mild diffuse degeneration in one fish from Wekiva and a minimal granuloma rating in one fish from Santa Fe.

Both head and hind kidneys were evaluated for pathologies, with parasites being the most prevalent pathology for both tissue types. Wekiva was found to have the highest incidence and degree of infestation. Other pathologies in Wekiva were limited to one fish with severe thrombotic lesions. Granulomas were found in 40% of St. Marys fish. Hind kidneys from Santa Fe fish had additional pathologies: hyaline droplet degeneration (15%) and renal casts (15 %).

Macrophage aggregates (MA) were analyzed and quantified for percent mm^2/tissue of MA, density/mm^2 and mean MA size (μm^2) within the spleen. Mean MA size and density was found to be higher in the fish from Wekiva, but was not significantly different (á=0.05) from the other sites. Fish from Santa Fe had the highest percent area of tissue with MA, but were not significantly different (á=0.05) from the other sites.

Santa Fe and St. Marys female LMB were found to be more reproductively mature than Wekiva. Of Wekiva females, 1 of 9 were in stage II or mid-development with the remaining 8

classified as stage III or late development. In contrast, St. Marys and Santa Fe females were more mature with the majority of fish classified as stage IV or late development/hydrated (5 and 6 respectively), with the remaining fish classified as stage III. All males from St. Marys and Santa Fe were found to be sexually mature (stage III or late spermatogenic). While males from Wekiva were predominately ripe (7 of 10), two males were classified as stage II or mid-spermatogenic and 1 male was considered stage I or early spermatogenic. Females used in microarray analysis of gene expression were selected to be at the same ovarian stage, stage III.

Percent atretic oocytes was calculated for all females. The more mature females from St. Marys and Santa Fe had significantly (á=0.05) more atretic oocytes than the females of Wekiva, with means of 29 % at St. Marys, 28 % at Santa Fe, and <1 % at Wekiva. Atresia among the St. Marys and Santa Fe females was generally higher for stage IV fish than for stage III fish. Santa Fe had the highest single rate of atresia with one female having 67% atretic oocytes.

Parasites were the only additional pathology found in the gonads of these fish. Wekiva had the highest rate of infestation with 10 of 19 fish (53%) having minimal to mild parasites. Males had the highest infestation rate with 80% of the testes having parasites. Santa Fe had no gonadal parasites, and St. Marys River had 2 fish (1 male and 1 female) with mild and minimal parasites found, respectively.

Supplemental Table S1 Gene set enrichment (GSEA) pathways affected in female largemouth bass (LMB) by experimental treatment of MeHg (Lab) and collected from a high MeHg environment (Field). Pathways were considered differentially affected when showing a median fold change greater than 10%. The number of measured entities in a pathway is reported, as well as the normalized enrichment score (ES), median fold change for the entire pathway, and the p-value.

	Name	***# of Measured Entities***	***Normalized ES***	***Median change***	***p-value***
Lab	Apoptosis	14	1.552	1.138	0.040
	Notch -> RBPJ/HES/HEY signaling	5	1.642	1.211	0.009
	GNRHR -> ELK-SRF signaling	13	1.526	1.241	0.028
	NeurotensinR -> ELK-SRF/AP-1/EGR signaling	18	1.595	1.263	0.027
	GFR -> NCOR2 signaling	18	1.463	1.263	0.049
	EGFR/ERBB2 -> HIF1A signaling	13	1.520	1.263	0.036
	OxytocinR -> ELK-SRF/GATA/AP-1 signaling	11	1.647	1.280	0.008
	EphrinB -> JUN signaling	8	1.569	1.484	0.048
	FrizzledR -> JUN/PAX2 signaling	5	1.681	1.485	0.009
Field	GHR -> ELK-SRF/MYC signaling	14	-1.584	-1.136	0.039
	Tricarboxylic acid cycle	16	1.476	1.175	0.037
	Melanogenesis	83	1.530	1.176	0.009
	Biosynthesis of cholesterol	12	1.603	1.178	0.013
	Actin Cytoskeleton Regulation	86	1.876	1.217	< 0.001
	EphrinR -> actin signaling	42	2.010	1.296	< 0.001

Supplemental Table S2 Sub network enrichment analysis (expression targets and binding partners) for neuroendocrine signaling and hormone receptors in the female largemouth bass (LMB) brain ($p<0.05$). Gene set seeds listed are those that had greater than 10 members in the network. The complete list of significantly regulated sub-networks is presented in Appendix 2. The table is arranged based upon the number of neighbors measured in the network.

Experiment	***Entity Relationship***	***Gene Set***	***# of Measured Neighbors***	***Median change***	***p-value***
Lab	Expression	paired-like homeodomain 2	11	-1.119	0.017
		colony stimulating factor 3 (granulocyte)	12	1.224	0.009
		homeo	13	-1.408	0.002
		myogenic differentiation 1	14	-1.122	0.026
		integrin	15	1.138	0.003
		activin	20	-1.256	0.001
		BCR-ABL	21	1.224	0.046
		GnRH1	27	-1.109	0.032
	Binding	Ras-related C3 botulinum toxin substrate 1	11	1.148	0.034
		tropomyosin	11	1.307	0.044
		v-src sarcoma	15	1.129	0.046
		beta-actin	15	1.132	0.030
		karyopherin alpha 2	17	1.160	0.031
Field	Expression	Ras-related C3 botulinum toxin substrate 1	10	1.570	0.031
		paired-like homeodomain 2	11	-1.187	0.008
		calcitonin/calcitonin-related polypeptide, alpha	11	1.252	0.032
		insulin-like growth factor 2 (somatomedin A)	11	1.570	0.016
		cyclin-dependent kinase inhibitor 1B	11	1.629	0.027
		sonic hedgehog	12	-1.187	0.040
		vasoactive intestinal peptide	12	1.121	0.023
		ciliary neurotrophic factor	12	1.334	0.014
		wingless-type MMTV integration site family, member 3A	12	1.507	0.009

		GATA binding protein 2	13	1.155	0.050
		Kruppel-like factor 4 (gut)	13	1.252	0.014
		calcium channel	13	1.474	0.031
		integrin	15	1.155	0.014
		activating transcription factor 2	16	1.458	0.007
		protein kinase C, alpha	18	1.573	0.049
		CREB binding protein	20	1.145	0.029
		mitogen-activated protein kinase kinase 1	20	1.327	0.029
		leukemia inhibitory factor	20	1.380	0.034
		SMAD family member	26	1.145	0.049
	Binding	histone H1	10	-1.289	0.017
		tight junction	10	1.801	0.023
		trans-golgi network protein 2	12	1.327	0.042
		vitamin D (1,25- dihydroxyvitamin D3) receptor	14	1.366	0.023
		focal adhesion	14	1.684	0.026
		actinin, alpha 1	16	1.433	0.026
		stress fibers	16	1.494	0.005

Supplemental Table S3 Biological processes that were over- or under-represented in female largemouth bass (LMB) brain in animals collected from the high MeHg site, St. Marys River. Class counts are the number of genes in the regulated list (Class 0) compared to the unregulated list with that biological process. Over/under indicates whether the biological process showed higher representation in the regulated list (over) or showed higher representation in the unregulated list (under). The complete list of gene ontology terms for both laboratory and field samples are provided in Appendix 2.

GO Biological Process	***Class 0 Count***	***Class 0 Total***	***Class 1 Count***	***Class 1 Total***	***p-value***	***over/under***
GO:0045941; positive regulation of transcription	4	2752	6	717	0.007	over
GO:0006457; protein folding	38	2752	20	717	0.013	over
GO:0051246; regulation of protein metabolic process	5	2752	6	717	0.014	over
GO:0006605; protein targeting	6	2752	6	717	0.022	over
GO:0006730; one-carbon compound metabolic process	6	2752	6	717	0.022	over
GO:0016071; mRNA metabolic process	6	2752	6	717	0.022	over
GO:0007018; microtubule-based movement	26	2752	1	717	0.029	under
GO:0006512; ubiquitin cycle	53	2752	24	717	0.031	over
GO:0008285; negative regulation of cell proliferation	17	2752	0	717	0.032	under
GO:0006811; ion transport	33	2752	2	717	0.033	under
GO:0007049; cell cycle	49	2752	5	717	0.041	under
GO:0006096; glycolysis	23	2752	1	717	0.043	under

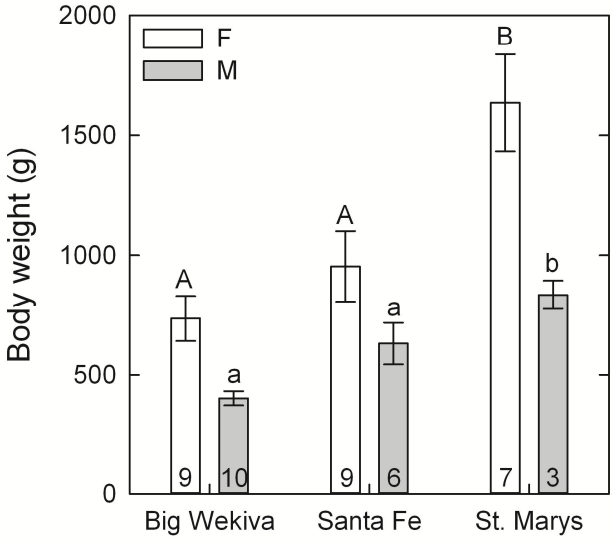

Fig. S1. Total body weights for female (F) and male (M) largemouth bass (LMB) collected at field sites. Columns with different letters denote significant differences (p=0.05).

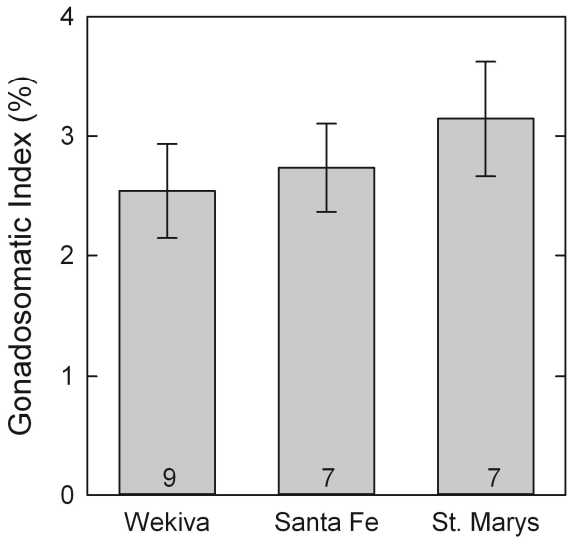

Fig. S2. Mean gonadosomatic index (GSI, %) for largemouth bass (LMB) collected at field sites. Columns with different letters denote significant differences (p=0.05).

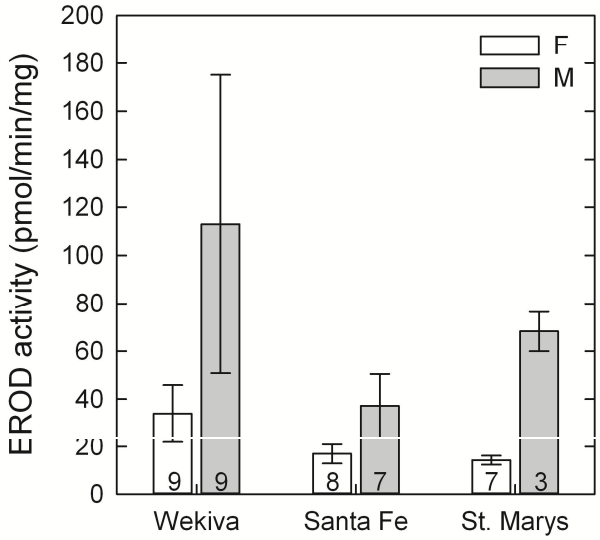

Fig. S3. Ethoxyresorufin-O-deethylase (EROD) activity in hepatic microsomes of female (F) and male (M) adult largemouth bass (LMB) collected from the Wekiva, Santa Fe, and St. Marys rivers. Error bars are one standard error of the mean. Number of fish assayed per group is labeled on each column. There were no significant differences between field sites.

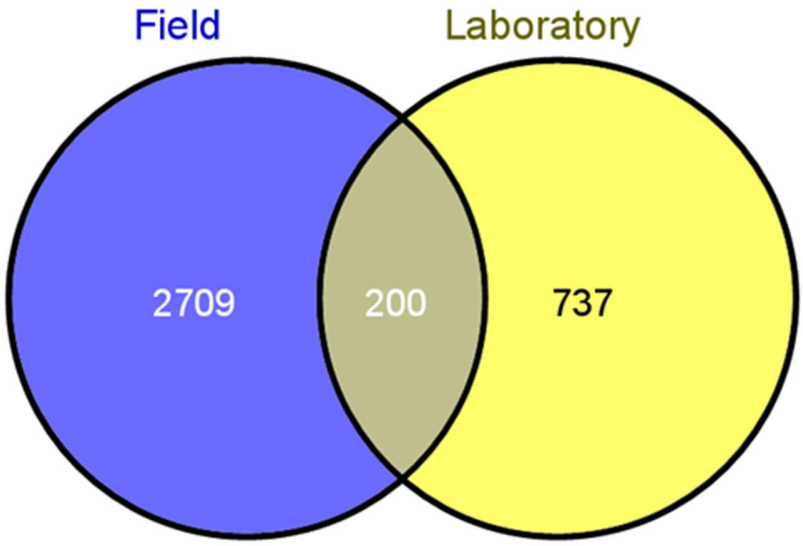

Figure S4 A Venn diagram showing the overlap in differentially expressed genes (raw $P < 0.05$) between laboratory and field samples.

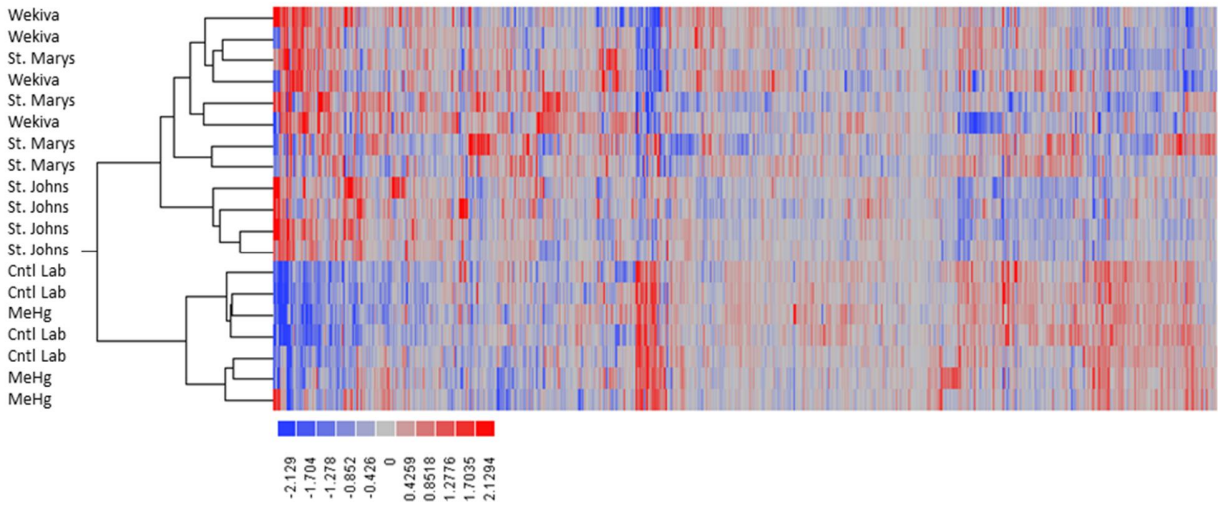

Fig. S5. Cluster analysis of gene expression patterns of female largemouth bass (LMB) hypothalamus from fish injected with MeHg and female LMB whole brain from animals collected from field sites in Florida. The laboratory and field samples separate into distinct clades. The LMB collected from St. Johns River also show a distinct separation from the other groups.

References

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