**Additional Material**

**Embryonic atrazine exposure and later in life behavioral and brain transcriptomic, epigenetic, and pathological alterations in adult male zebrafish**

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**Supplemental Methods**

*Evaluation of Global Brain Methylation*

The zebrafish brains were homogenized in lysis buffer (50 mM Tris, 100 mM EDTA, 100 mM NaCl, 1% SDS, 100 µg/mL Proteinase K) and incubated overnight at 55°C with rocking agitation. The following day, samples were transferred to 1.5 Phase Lock Gel (PLG; QuantaBio, Beverly, MA) tubes. Phenol (phenol-Tris saturated, pH 8; Roche, Indianapolis, IN) and chloroform:isoamyl alcohol (American Bioanalytical, Natick, MA) were added and the samples centrifuged at room temperature for 5 minutes at 1500 rcf. The upper aqueous phase was transferred to a new PLG tube and the previous steps repeated. The upper aqueous phase was transferred to a 2 ml screw top microcentrifuge tube (Fisher Scientific, Hampton, NH) and 0.1X volume of 3M sodium acetate and 1X volume of isopropanol added and the tube inverted until the DNA started to aggregate. The samples were incubated at room temperature and then centrifuged at 4°C for 10 minutes at 800 rcf to form a DNA pellet. The pellet was washed in 70% ethanol twice, centrifuging at 4°C for 10 minutes at 800 rcf between washes. After the second wash, the pellet was centrifuged again and the sample allowed to dry to remove remaining ethanol. The sample was rehydrated in 1xTLE buffer and incubated overnight at 55°C with rocking agitation. The following morning the DNA quality and concentration was checked with a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE) and stored at 4°C until further analysis.

**Table S1. Primers used in qPCR analysis**

|  |  |  |
| --- | --- | --- |
| Seq ID | Gene Symbol | Primer Sequences |
| NM\_181601 | *β-actin* | ctaaaaactggaacggtgaagg |
|  |  | aggcaaataagtttcggaacaa |
| NM\_207059.1 | *aqp1a* | ggctgcgctaatctatgacttt |
|  |  | gggtcactttgaggacatctct |
| NM\_131719.1 | *cdk5* | cggatgtactgtttggtgctaa |
|  |  | gtctgccactgttcttctgttg |
| NM\_212666.1 | *cyp26b1* | tatccttcaacatggctgtacg |
|  |  | ggagtgagtctcttgctcgaat |
| NM\_212924.2 | *igfbp7* | tctgatctcaccgctcactaaa |
|  |  | caccatctggacaggaatatca |
| NM\_199980.1 | *itm2cb* | gaaatccaaatgccgtactctc |
|  |  | atccgacagtggaagaaactgt |
| NM\_198914.2 | *sult2* | cacagctcgagtttctgctcta |
|  |  | gcctgatttagggaaagtgatg |

**Table S2. Top pathways altered by embryonic 0.3 ppb ATZ exposure**

|  |  |  |
| --- | --- | --- |
| Diseases and Disorders | | |
| Name | p-valuea | # Moleculesb |
| Cancer | 9.23E-03 - 5.20E-09 | 115 |
| Organismal Injury and Abnormalities | 9.78E-03 - 5.20E-09 | 116 |
| Gastrointestinal Disease | 8.24E-03 - 1.42E-08 | 108 |
| Endocrine System Disease | 9.14E-03 - 2.65E-06 | 44 |
| Reproductive System Disease | 7.47E-03 - 2.65E-06 | 64 |
| Molecular and Cellular Functions | | |
| Name | p-valuea | # Moleculesb |
| Cell Morphology | 7.96E-03 - 1.66E-05 | 31 |
| Cellular Compromise | 5.16E-03 - 1.66E-05 | 12 |
| Cellular Development | 9.11E-03 - 1.30E-04 | 24 |
| Cellular Growth and Proliferation | 7.33E-03 - 1.30E-04 | 23 |
| Cell Cycle | 9.73E-03 - 2.20E-04 | 23 |
| Physiological System Development and Function | | |
| Name | p-valuea | # Moleculesb |
| Organ Morphology | 8.53E-03 - 2.65E-06 | 33 |
| Reproductive System Development and Function | 7.25E-03 - 2.65E-06 | 22 |
| Tissue Morphology | 7.31E-03 - 7.02E-06 | 41 |
| Connective Tissue Development and Function | 8.24E-03 - 3.43E-05 | 21 |
| Organismal Development | 9.78E-03 - 5.22E-05 | 54 |

aDerived from the likelihood of observing the degree of enrichment in a gene set of a given size by chance alone.

bClassified as being differentially expressed that relate to the specified function category; a gene may be present in more than one category.

**Table S3. Top pathways altered by embryonic 3 ppb ATZ exposure**

|  |  |  |
| --- | --- | --- |
| Diseases and Disorders | | |
| Name | p-valuea | # Moleculesb |
| Connective Tissue Disorders | 1.19E-02 - 1.12E-06 | 90 |
| Organismal Injury and Abnormalities | 1.19E-02 - 3.15E-05 | 88 |
| Skeletal and Muscular Disorders | 1.19E-02 - 7.71E-05 | 30 |
| Reproductive System Disease | 1.19E-02 - 1.15E-04 | 78 |
| Endocrine System Disorders | 1.19E-02 - 1.57E-04 | 7 |
| Molecular and Cellular Functions | | |
| Name | p-valuea | # Moleculesb |
| Cellular Development | 1.19E-02 - 4.83E-05 | 22 |
| Molecular Transport | 1.19E-02 - 9.44E-05 | 10 |
| Cellular Growth and Proliferation | 1.19E-02 - 1.03E-04 | 20 |
| Cellular Movement | 1.08E-02 - 3.69E-04 | 26 |
| Cell-to-Cell Signaling and Interaction | 1.19E-02 - 4.51E-04 | 11 |
| Physiological System Development and Function | | |
| Name | p-valuea | # Moleculesb |
| Organismal Development | 1.19E-02 - 3.89E-07 | 32 |
| Embryonic Development | 1.19E-02 - 4.90E-07 | 28 |
| Digestive System Development and Function | 1.19E-02 - 1.84E-06 | 14 |
| Nervous System Development and Function | 1.19E-02 - 6.55E-06 | 25 |
| Organ Development | 1.19E-02 - 6.55E-06 | 29 |

aDerived from the likelihood of observing the degree of enrichment in a gene set of a given size by chance alone.

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bClassified as being differentially expressed that relate to the specified function category; a gene may be present in more than one category.

**Table S4. Top pathways altered by embryonic 30 ppb ATZ exposure**

|  |  |  |
| --- | --- | --- |
| Diseases and Disorders | | |
| Name | p-valuea | # Moleculesb |
| Cancer | 1.48E-02 - 2.77E-07 | 113 |
| Organismal Injury and Abnormalities | 1.48E-02 - 2.77E-07 | 114 |
| Gastrointestinal Disease | 1.03E-02 - 9.50E-06 | 103 |
| Renal and Urological Disease | 1.03E-02 - 9.94E-05 | 31 |
| Hereditary Disorder | 1.48E-02 - 3.17E-04 | 42 |
| Molecular and Cellular Functions | | |
| Name | p-valuea | # Moleculesb |
| Amino Acid Metabolism | 1.03E-02 - 2.64E-05 | 12 |
| Small Molecule Biochemistry | 1.25E-02 - 2.64E-05 | 33 |
| Molecular Transport | 1.25E-02 - 7.07E-05 | 20 |
| Cell-to-Cell Signaling and Interaction | 1.24E-02 - 2.25E-05 | 13 |
| Cellular Assembly and Organization | 1.24E-02 - 2.25E-05 | 22 |
| Physiological System Development and Function | | |
| Name | p-valuea | # Moleculesb |
| Tissue Development | 1.26E-02 - 1.03E-04 | 24 |
| Tissue Morphology | 1.35E-02 - 1.29E-04 | 37 |
| Humoral Immune Response | 1.48E-02 - 2.61E-04 | 3 |
| Cardiovascular System Development and Function | 1.16E-02 - 3.15E-04 | 23 |
| Organ Morphology | 1.45E-02 - 3.91E-04 | 24 |

aDerived from the likelihood of observing the degree of enrichment in a gene set of a given size by chance alone.

bClassified as being differentially expressed that relate to the specified function category; a gene may be present in more than one category.

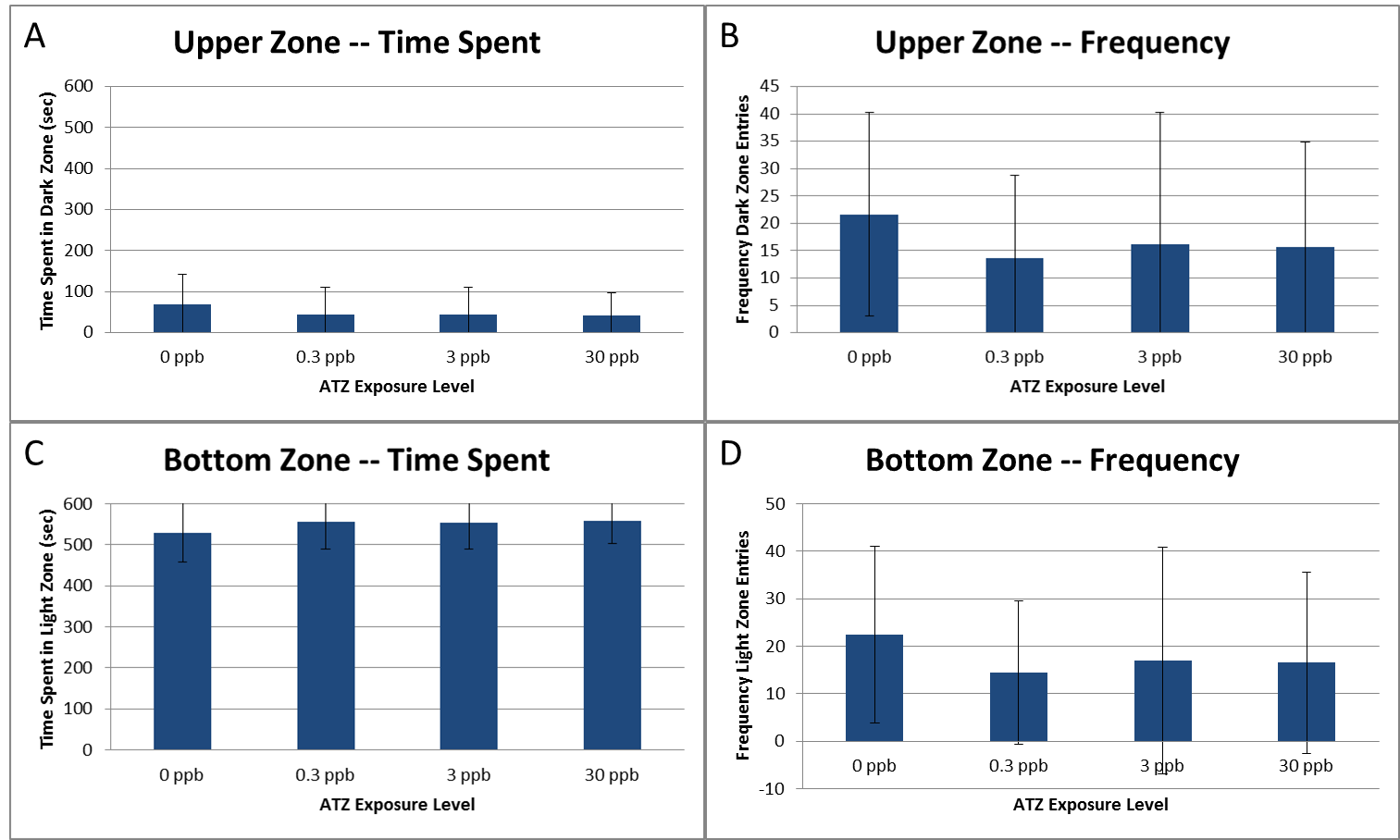
**Table S5 List of genes with altered expression in males at 6 and 9 mpf by embryonic exposure.**

|  |  |  |
| --- | --- | --- |
| Name | Gene Symbola | Biological Functionb |
| Genes altered in 0.3 ppb exposure at 6 mpf and 9 mpf | | |
| Beta-1,3-glucuronyl-transferase 3 | *B3GAT3* | Carbohydrate metabolic process; chondroitin sulfate metabolic process; glycosaminoglycan biosynthesis; protein glycosylation |
| Bruton tyrosine kinase | *BTK* | Adaptive immune response; apoptotic process; B cell activation, affinity maturation and receptor signaling; mast cell histamine release |
| Caveolae associated protein 4 | *CAVIN4* | Cardiac myofibril assembly; cell differentiation; muscle development; regulation of gene expression; Rho protein signal transduction |
| Cyclin dependent kinase 5 | *CDK5* | Associative learning; dopaminergic and glutamatergic synaptic transmission; neuron and oligodendrocyte development and differentiation |
| Complement factor H | *CFH* | Cellular response to hydrogen peroxide, interferon-gamma, and lipopolysaccharide; complement activation; regulation of cytolysis |
| Carboxypeptidase M | *CPM* | Anatomical structure morphogenesis; C-terminal protein lipidation; peptide metabolic process; protein processing; proteolysis |
| Cytochrome P450, family 2, subfamily ac, peptide 1 | *CYP2AC1* | -- |
| Eosinophil peroxidase | *EPX* | Defense response; eosinophil migration; Regulation of interleukin-10, interleukin-5, and interleukin-4 production |
| Glutamic--pyruvic transaminase | *GPT* | Biosynthetic process; cellular amino acid biosynthetic process; L-alanine catabolic process |
| Hypoxia inducible factor 3 alpha subunit | *HIF3A* | Angiogenesis; apoptotic process; cellular response to hypoxia; protein ubiquitination; regulation of transcription |
| Hydroxysteroid 11-beta dehydrogenase 2 | *HSD11B2* | Glucocorticoid biosynthesis and metabolism; response to glucocorticoid, hypoxia, insulin, and steroid hormone stimulus |
| HtrA serine peptidase 2 | *HTRA2* | Adult locomotor behavior; response to herbicide; forebrain development; negative regulation of cell cycle; neuron development; proteolysis |
| Interferon induced protein 44 | *IFI44* | Response to virus |
| Insulin like growth factor binding protein 7 | *IGFBP7* | Cell adhesion; cellular response to hormone stimulus; post-translational protein modification; regulation of steroid biosynthesis |
| Integral membrane protein 2C | *ITM2C* | Negative regulation of amyloid precursor protein; nervous system development and differentiation; regulation of apoptosis |
| Kynurenine 3-monooxygenase | *KMO* | Kynurenine metabolism; NAD biosynthesis and metabolism; pyridine biosynthesis; quinolinate biosynthesis; tryptophan catabolism |
| L-2-hydroxyglutarate dehydrogenase | *L2HGDH* | 2-oxoglutarate metabolic process; cellular protein metabolic process; oxidation-reduction process |
| Membrane palmitoylated protein 1 | *MPP1* | GDP metabolic process; GMP metabolic process; regulation of neutrophil chemotaxis; signal transduction |
| Protein disulfide isomerase family A member 6 | *PDIA6* | Apoptotic cell clearance; cell redox homeostasis; platelet activation and aggregation; post-translational protein modification and folding |
| Protein phosphatase Mg2+/Mn2+ dependent 1L | *PPM1L* | MAPK cascade; protein dephosphorylation; sphingolipid biosynthesis; transmembrane receptor protein serine/threonine kinase signaling |
| Protein regulator of cytokinesis 1 | *PRC1* | Cell cycle; cell division; cytokinesis; microtubule bundle formation; microtubule cytoskeleton organization; mitotic spindle elongation |
| Pleckstrin and Sec7 domain containing 3 | *PSD3* | ARF protein signal transduction; positive regulation of GTPase activity; regulation of ARF protein signal transduction |
| Solute carrier family 43 member 1 | *SLC43A1* | Amino acid transport; L-amino acid transport; neutral amino acid transport; transmembrane transport; transport |
| Sp8 transcription factor | *SP8* | Dorsal/ventral and proximal/distal pattern formation; embryonic limb morphogenesis; regulation of transcription |
| Sulfotransferase family 2B member 1 | *SULT2B1* | Sulfate conjugation of hormones, xenobiotic compounds, and neurotransmitters; lipid metabolic process; steroid metabolic process |
| Troponin I2, fast skeletal type | *TNNI2* | Regulation of muscle contraction; co-activator of estrogen receptor-related receptor alpha; positive regulation of transcription |
| Zinc finger protein 729 | *ZNF729* | Regulation of transcription |
| Genes altered in 3 ppb exposure at 6 mpf and 9 mpf | | |
| Aminoacylase 1 | *ACY1* | Cellular amino acid metabolic process; protein catabolic process; proteolysis; xenobiotic metabolic process |
| Caveolae associated protein 4 | *CAVIN4* | Cardiac myofibril assembly; cell differentiation; muscle development; regulation of gene expression; Rho protein signal transduction |
| Cyclin dependent kinase 5 | *CDK5* | Associative learning; dopaminergic and glutamatergic synaptic transmission; neuron and oligodendrocyte development and differentiation |
| Cytochrome P450, family 2, subfamily ac, peptide 1 | *CYP2AC1* | -- |
| Cytochrome P450 family 2 subfamily J member 2 | *CYP2J2* | Arachidonic acid, icosanoid, and linoleic acid metabolism; negative regulation of collagen biosynthesis; xenobiotic metabolic process |
| Distal-less homeobox 2 | *DLX2* | Hippocampus, subpallium, olfactory bulb, and cerebral cortex development; nerve morphogenesis; oligodendrocyte differentiation |
| EH domain binding protein 1 like 1 | *EHBP1l1* | Rab effector protein; vesicle trafficking |
| Erb-b2 receptor tyrosine kinase 4 | *ERBB4* | Apoptotic process; cell fate; cell migration; cell proliferation; central nervous system morphogenesis; synapse maturation |
| Coagulation factor X | *F10* | Blood coagulation; ER to Golgi vesicle-mediated transport; peptidyl-glutamic acid carboxylation; protein kinase B signaling cascade |
| GSH2, GS homeobox 2 | *GSX2* | Central nervous system development; forebrain and hindbrain morphogenesis; neuron fate; oligodendrocyte differentiation |
| Histidine decarboxylase | *HDC* | Carboxylic acid metabolism; catecholamine biosynthesis; cellular amino acid metabolism; histamine biosynthesis and metabolism |
| HtrA serine peptidase 2 | *HTRA2* | Adult locomotor behavior; response to herbicide; forebrain development; negative regulation of cell cycle; neuron development; proteolysis |
| Insulin like growth factor binding protein 7 | *IGFBP7* | Cell adhesion; cellular response to hormone stimulus; post-translational protein modification; regulation of steroid biosynthesis |
| Integral membrane protein 2C | *ITM2C* | Negative regulation of amyloid precursor protein; nervous system development and differentiation; regulation of apoptosis |
| N-acetyltransferase 1 | *NAT1* | Digestive tract development; response to hypoxia; response to lipopolysaccharide; xenobiotic metabolic process |
| Protein disulfide isomer-ase family A member 6 | *PDIA6* | Apoptotic cell clearance; cell redox homeostasis; platelet activation and aggregation; post-translational protein modification and folding |
| Ribonuclease P/MRP subunit p21 | *RPP21* | Response to drug; rRNA processing; tRNA 5'-leader removal; tRNA processing |
| Solute carrier family 5 member 1 | *SLC5A1* | Carbohydrate and glucose transport; intestinal absorption; intestinal glucose absorption; ion transport; metanephros development |
| Sp8 transcription factor | *SP8* | Dorsal/ventral and proximal/distal pattern formation; embryonic limb morphogenesis; regulation of transcription |
| Sulfotransferase family 2B member 1 | *SULT2B1* | Sulfate conjugation of hormones, xenobiotic compounds, and neurotransmitters; lipid metabolic process; steroid metabolic process |
| Tryptophan 2,3-dioxygenase | *TDO2* | Oxidation-reduction process; protein homotetramerization; tryptophan catabolism to acetyl-CoA and to kynurenine |
| Troponin I2, fast skeletal type | *TNNI2* | Regulation of muscle contraction; co-activator of estrogen receptor-related receptor alpha; positive regulation of transcription |
| Zinc finger protein 385B | *ZNF385B* | Apoptotic process; signal transduction by p53 class mediator resulting in induction of apoptosis |
| Genes altered in 30 ppb exposure at 6 mpf and 9 mpf | | |
| Aminoacylase 1 | *ACY1* | Cellular amino acid metabolic process; protein catabolic process; proteolysis; xenobiotic metabolic process |
| Adenylate kinase 7 | *AK7* | Axoneme assembly; brain development; cell projection organization; inflammatory response to antigenic stimulus; spermatogenesis |
| ATPase Na+/K+ trans-porting subunit beta 1 | *ATP1B1* | ATP metabolic process; blastocyst development; cell communication; cellular calcium and potassium ion homeostasis; metal ion transport |
| Beta-1,3-glucuronyltransferase 3 | *B3GAT3* | Carbohydrate metabolic process; chondroitin sulfate metabolic process; glycosaminoglycan biosynthesis; protein glycosylation |
| Bruton tyrosine kinase | *BTK* | Adaptive immune response; apoptotic process; B cell activation, affinity maturation and receptor signaling; mast cell histamine release |
| Caveolae associated protein 4 | *CAVIN4* | Cardiac myofibril assembly; cell differentiation; muscle development; regulation of gene expression; Rho protein signal transduction |
| Cyclin dependent kinase 5 | *CDK5* | Associative learning; dopaminergic and glutamatergic synaptic transmission; neuron and oligodendrocyte development and differentiation |
| Carboxypeptidase M | *CPM* | Anatomical structure morphogenesis; C-terminal protein lipidation; peptide metabolic process; protein processing; proteolysis |
| Dystrobrevin beta | *DTNB* | Metal ion binding; protein binding; zinc ion binding |
| Engrailed homeobox 1 | *EN1* | Cerebellum, mid-, and hind brain development; dopaminergic neuron differentiation; neuron development; locomotor and social behavior |
| Eosinophil peroxidase | *EPX* | Defense response; eosinophil migration; Regulation of interleukin-10, interleukin-5, and interleukin-4 production |
| Family with sequence similarity 43 member B | *FAM43B* | Negative regulation of cell proliferation |
| Glutamic--pyruvic transaminase | *GPT* | Biosynthetic process; cellular amino acid biosynthetic process; L-alanine catabolic process |
| Hemoglobin subunit epsilon 1 | *HBE1* | Blood coagulation; oxygen transport; response to organic cyclic compound |
| Hypoxia inducible factor 3 alpha subunit | *HIF3A* | Angiogenesis; apoptotic process; cellular response to hypoxia; protein ubiquitination; regulation of transcription |
| Hydroxysteroid 11-beta dehydrogenase 2 | *HSD11B2* | Glucocorticoid biosynthesis and metabolism; response to glucocorticoid, hypoxia, insulin, and steroid hormone stimulus |
| HtrA serine peptidase 2 | *HTRA2* | Adult locomotor behavior; response to herbicide; forebrain development; negative regulation of cell cycle; neuron development; proteolysis |
| Insulin like growth factor binding protein 7 | *IGFBP7* | Cell adhesion; cellular response to hormone stimulus; post-translational protein modification; regulation of steroid biosynthesis |
| Integral membrane protein 2C | *ITM2C* | Negative regulation of amyloid precursor protein; nervous system development and differentiation; regulation of apoptosis |
| L-2-hydroxyglutarate dehydrogenase | *L2HGDH* | 2-oxoglutarate metabolic process; cellular protein metabolic process; oxidation-reduction process |
| Membrane palmitoylated protein 1 | *MPP1* | GDP metabolic process; GMP metabolic process; regulation of neutrophil chemotaxis; signal transduction |
| Peroxiredoxin 3 | *PRDX3* | Cell redox homeostasis; regulation of proliferation; myeloid cell differentiation; regulation of neuron apoptotic process  217 |
| Protein kinase C delta | *PRKCD* | Phospholipase C and protein kinase activity; regulation of glial apoptosis; interleukin-10 and -12 production; histone phosphorylation |
| Protein kinase C epsilon | *PRKCE* | Apoptotic process; cell cycle; locomotory exploration behavior; protein ubiquitination; regulation of GABAergic synaptic transmission |
| Solute carrier family 43 member 1 | *SLC43A1* | Amino acid transport; L-amino acid transport; neutral amino acid transport; transmembrane transport; transport |
| Slingshot protein phosphatase 1 | *SSH1* | Actin cytoskeleton organization; regulation of excitatory postsynaptic membrane potential; regulation of synaptic plasticity; axonogenesis |
| Sulfotransferase family 2B member 1 | *SULT2B1* | Sulfate conjugation of hormones, xenobiotic compounds, and neurotransmitters; lipid metabolic process; steroid metabolic process |
| Tryptophan 2,3-dioxygenase | *TDO2* | Oxidation-reduction process; protein homotetramerization; tryptophan catabolism to acetyl-CoA and to kynurenine |
| Troponin I2, fast skeletal type | *TNNI2* | Regulation of muscle contraction; co-activator of estrogen receptor-related receptor alpha; positive regulation of transcription |

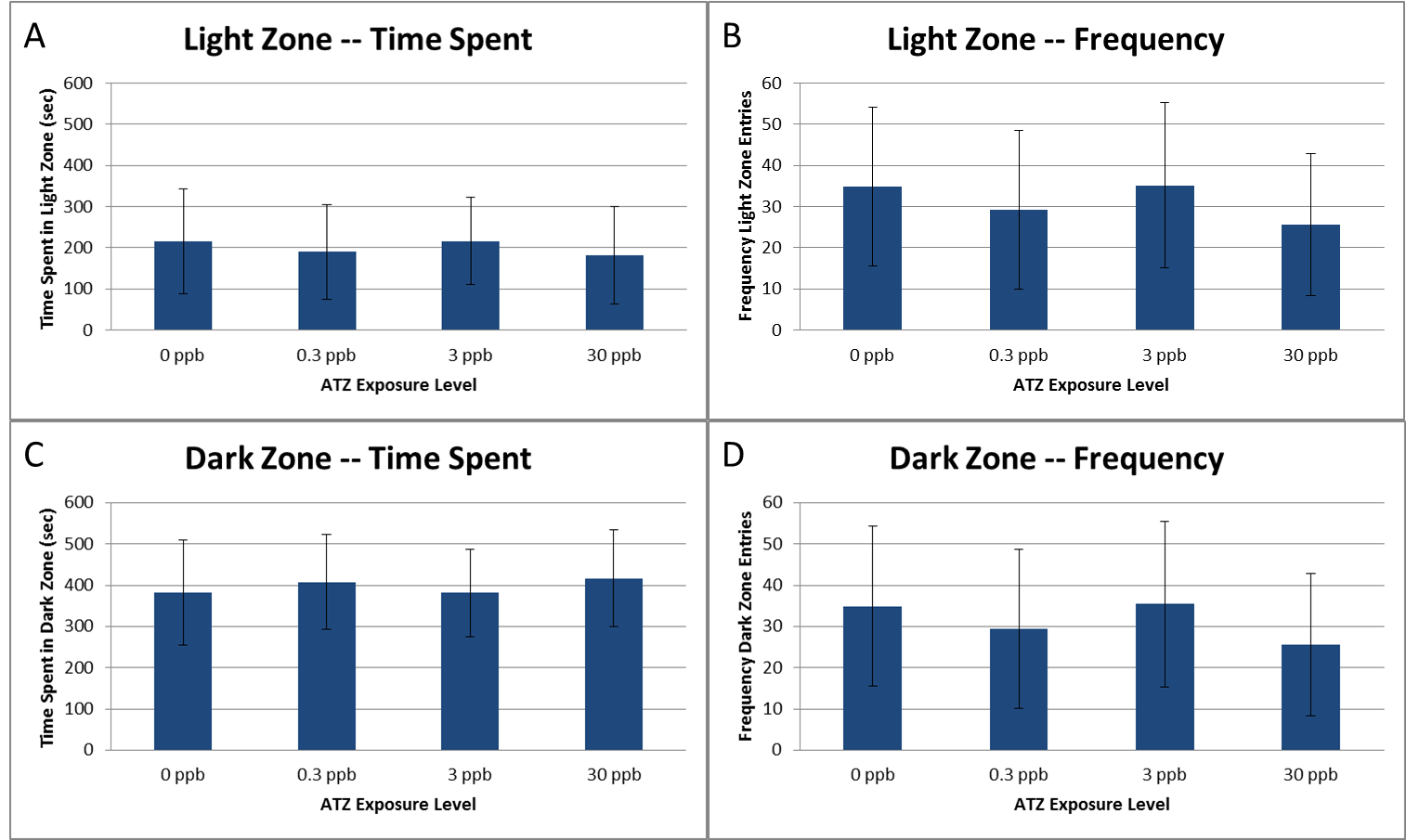
a Human ortholog of zebrafish gene

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b Determined via Ingenuity Pathway Analysis

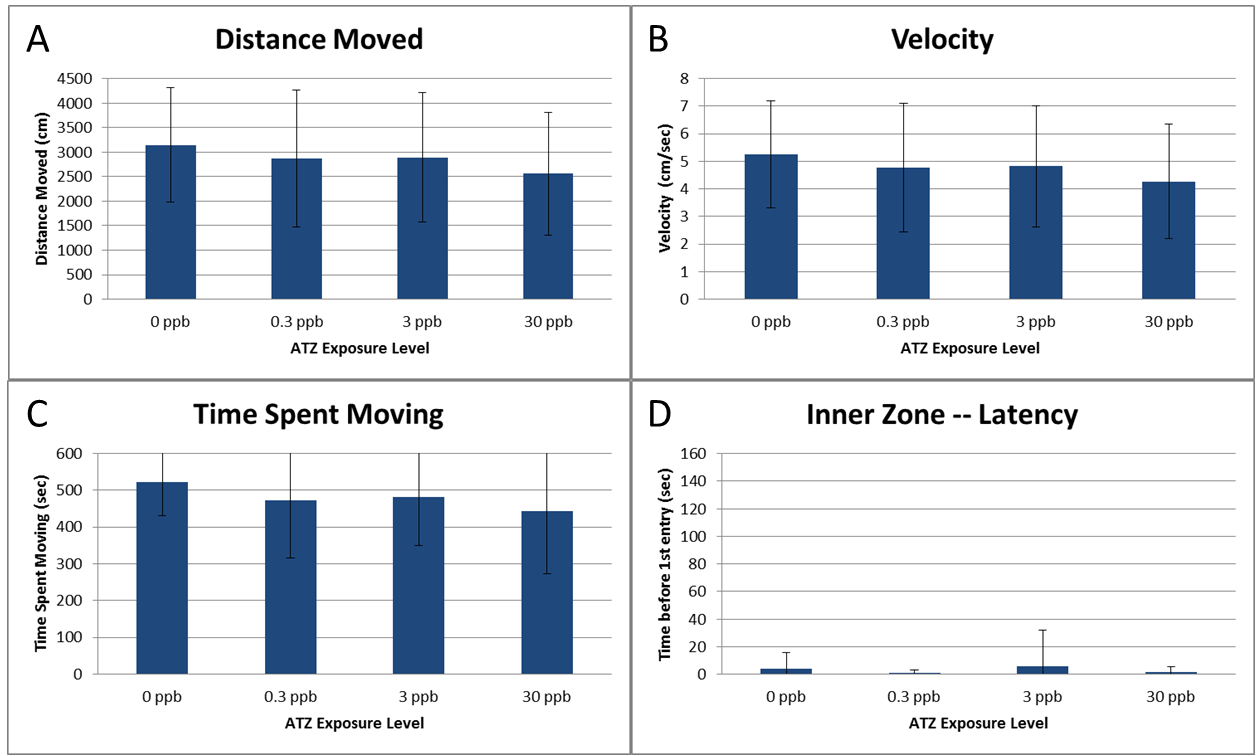


**Figure S1. Additional NTT results from male zebrafish with embryonic ATZ exposure.** There was no significant difference in the time spent in the upper zone (A), frequency of upper zone entries (B), time spent in the bottom zone (C), or frequency of bottom zone entries (D). N = 4, 10 subsamples per treatment per replicate to total 40 fish per treatment, error bars represent standard deviation, p>0.05.

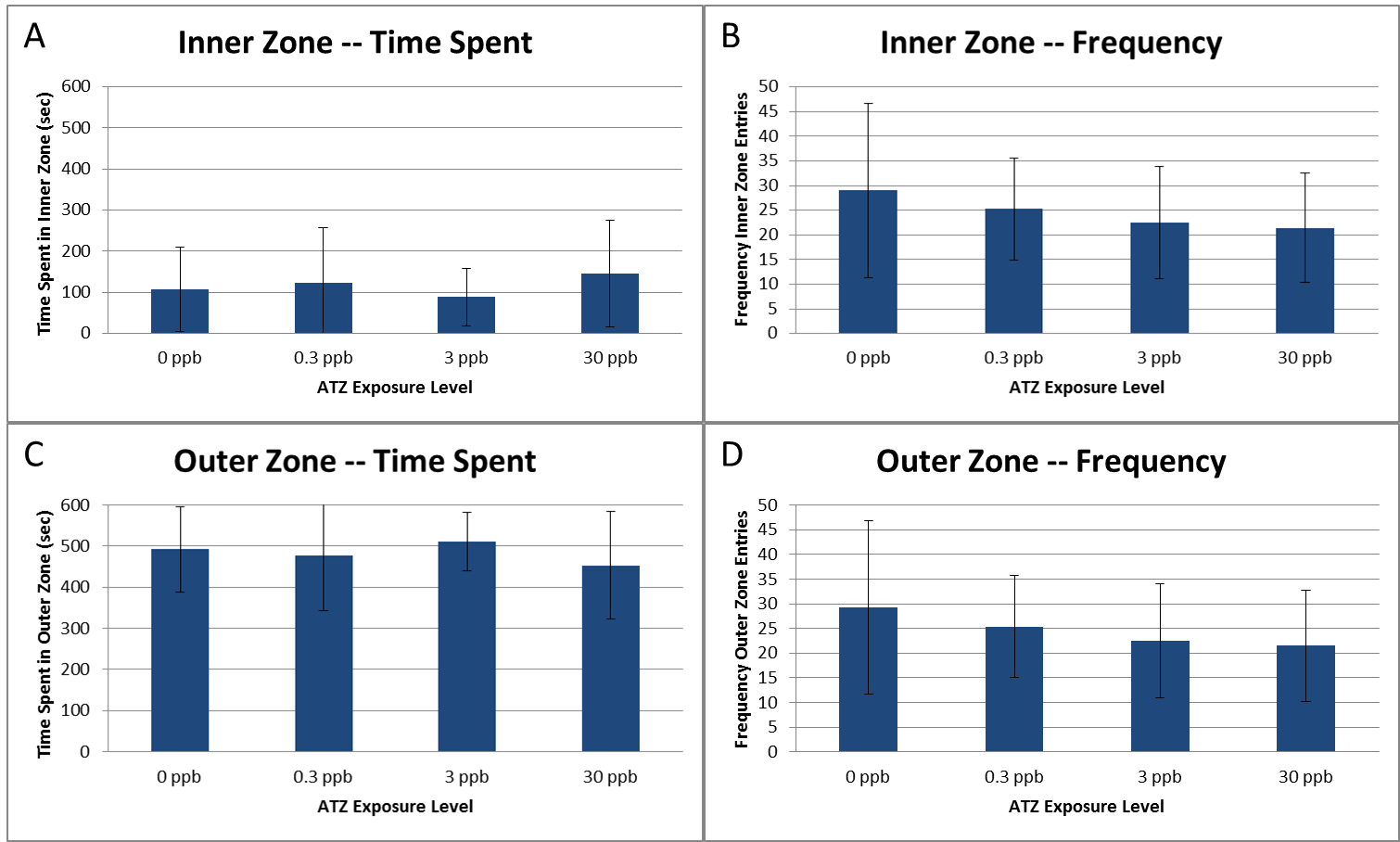


**Figure S2. Additional LDB results from male zebrafish with embryonic ATZ exposure.** There was no significant difference in the time spent in the light zone (A), frequency of light zone entries (B), time spent in the dark zone (C), or frequency of dark zone entries (D). N = 4, 10 subsamples per treatment per replicate to total 40 fish per treatment, error bars represent standard deviation, p>0.05.

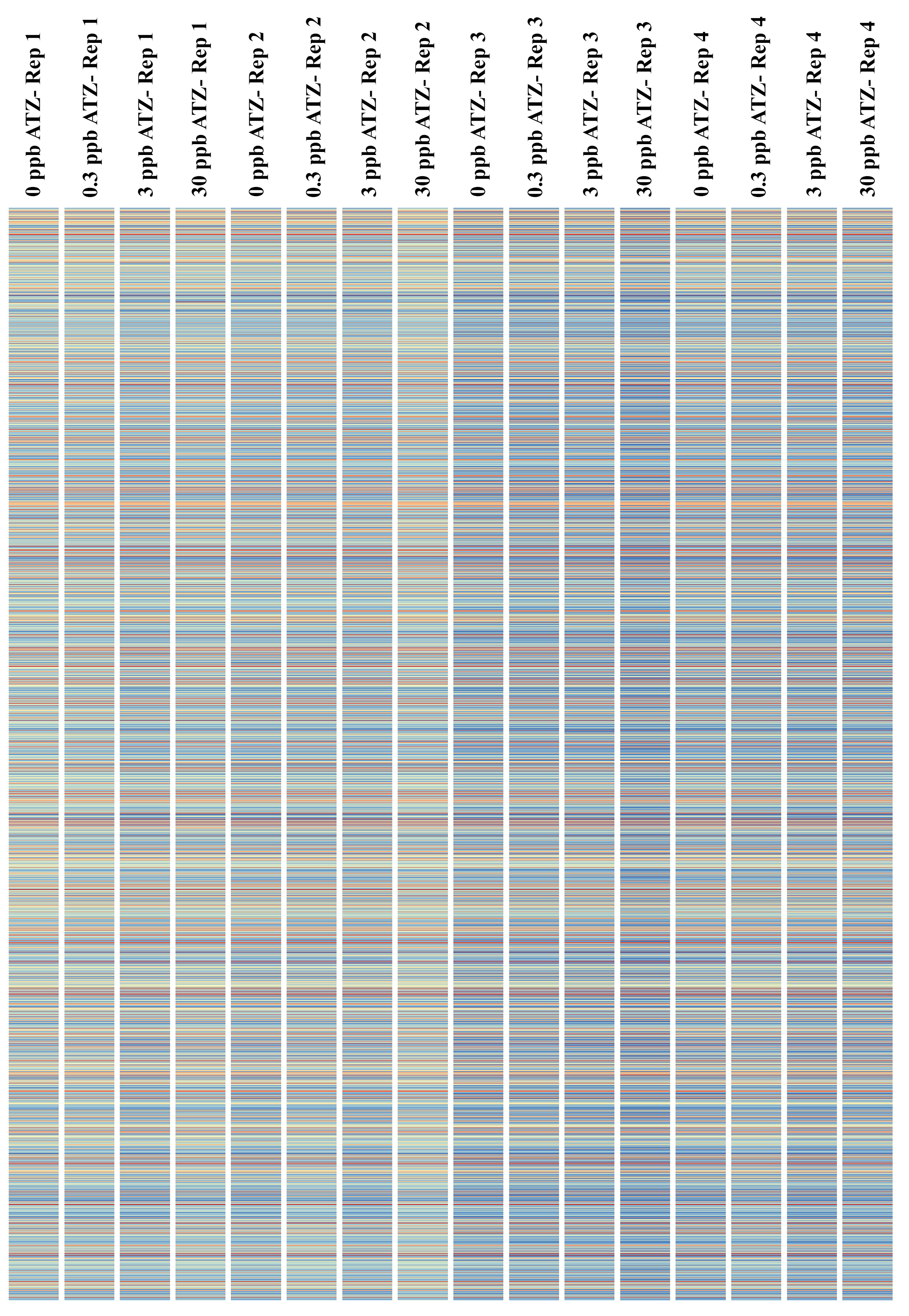
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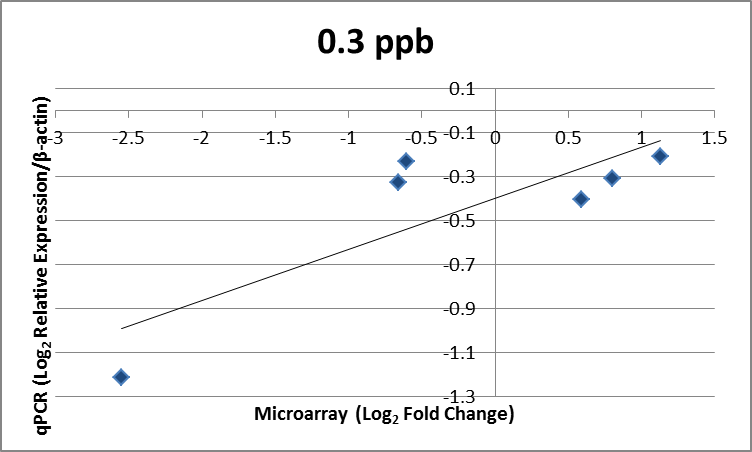
**Figure S3. OFT performance of male zebrafish with embryonic ATZ exposure.** Male zebrafish had a trend towards decreasing activity with increasing embryonic ATZ exposure, however, it did not reach significance for distance moved (A), velocity (B), or time spent moving (C). There was no significant differences in the latency to the first inner zone entry (D) between treatments. N = 4, 10 subsamples per treatment per replicate to total 40 fish per treatment, error bars represent standard deviation, p>0.05.

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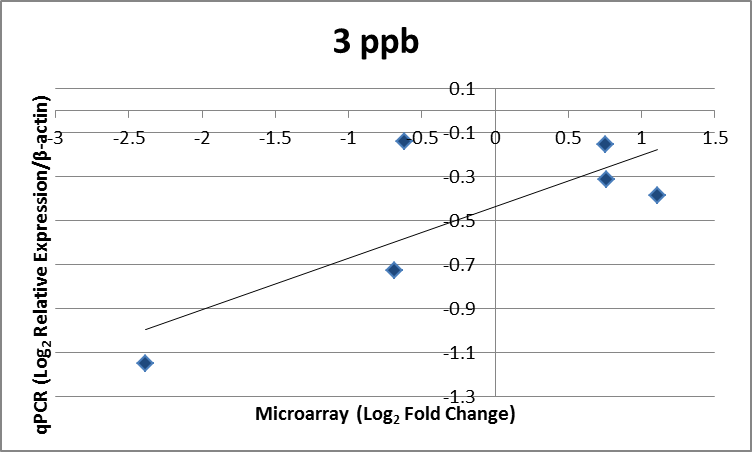
**Figure S4. Additional LDB results from male zebrafish with embryonic ATZ exposure.** There was no significant difference in the time spent in the light zone (A), frequency of light zone entries (B), time spent in the dark zone (C), or frequency of dark zone entries (D). N = 4, 10 subsamples per treatment per replicate to total 40 fish per treatment, error bars represent standard deviation, p>0.05



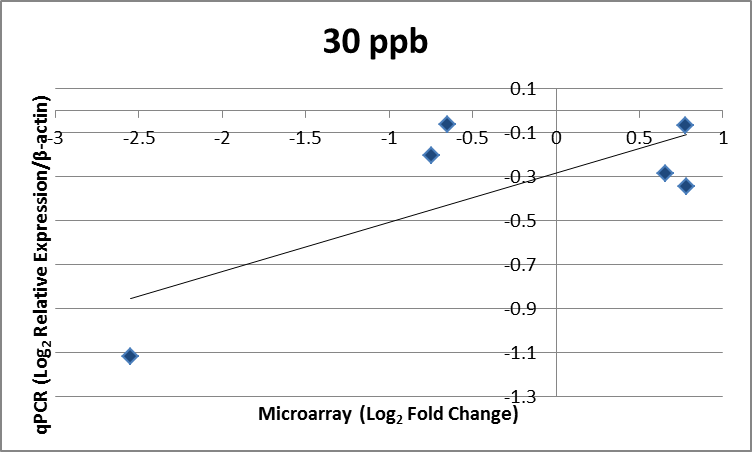
**Figure S5. Heat map of the male brain microarray results.** N = 4.



A



B

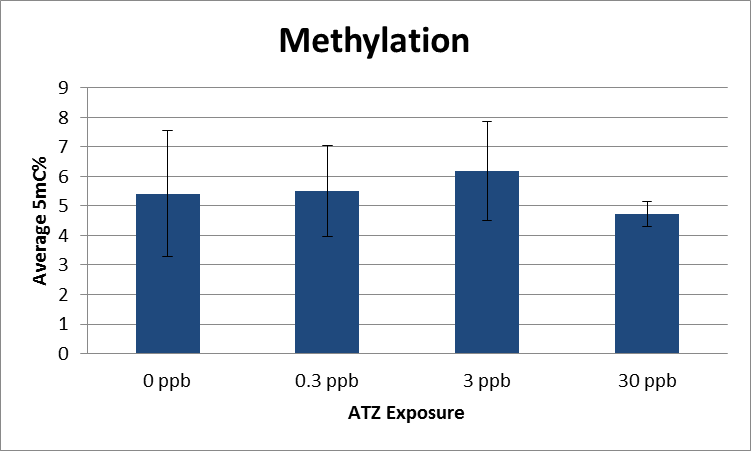


C

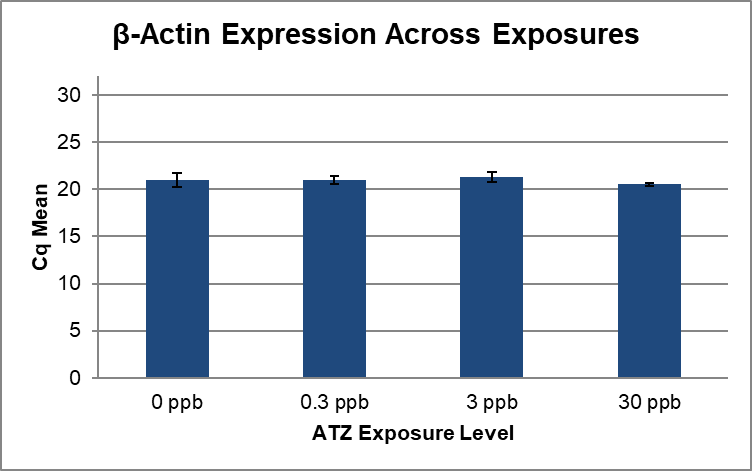
**Figure S6. Correlation between qPCR relative expression and microarray fold change in males.** There was a strong positive correlation between the log2 relative expression determined by qPCR and the microarray log2 fold change for all treatments (0.3 ppb: r=0.8285; 3 ppb: r=0.7875; 30 ppb: r=0.7466). The correlation was significant for males with embryonic exposure to 0.3 (A), 3 (B), and 30 ppb (C) ATZ (p<0.05). N = 4, 6 genes were evaluated.

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**Figure S7. Representative image of zebrafish brain used for morphometric evaluation.** Green area outlines the dorsal telencephalon. The yellow labels the posterior tuberculum. The red outlines a portion of the raphe populations (superior raphe does not appear in this plane of section). Scale bar represents 600 µm.



**Figure S8. Global methylation of male zebrafish with embryonic ATZ exposure.** There was no significant difference in percent 5mC between exposure groups (p>0.05). N=6-7.



**Figure S9. β-actin expression across ATZ exposure samples.** In a pre-analysis assessment, several reference genes were first tested to determine, which was the most consistently expressed among our samples. The expression of β-actin was not significantly different between ATZ exposures (p=0.1632) and was identified as the most consistently expressed among our samples. Thus, β-actin was included as the reference gene in the qPCR analysis. N = 4, qPCR run in triplicate, error bars represent standard deviation.