Symptomatic and asymptomatic domoic acid exposure in zebrafish (*Danio rerio*) revealed distinct non-overlapping gene expression patterns in the brain

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Key words: Domoic acid; harmful algal bloom toxin; glutamate; excitotoxin

Abstract

Domoic acid (DA) is a naturally produced neurotoxin synthesized by marine diatoms in the genus *Pseudo-nitzschia.* DA accumulates in filter-feeders such as shellfish, and can cause severe neurotoxicity when contaminated seafood is ingested, resulting in Amnesic Shellfish Poisoning (ASP) in humans. Overt clinical signs of neurotoxicity include seizures and disorientation. ASP is a significant public health concern, and though seafood regulations have effectively minimized the human risk of severe acute DA poisoning, the effects of exposure at asymptomatic levels are poorly understood. The objective of this study was to determine the effects of exposure to symptomatic and asymptomatic doses of DA on gene expression patterns in the zebrafish brain. We exposed adult zebrafish to either a symptomatic (1.1 ± 0.2 μg DA/g fish) or an asymptomatic (0.31 ± 0.03 μg DA/g fish) dose of DA by intracelomic injection and sampled at 24, 48 and 168 h post-injection. Transcriptional profiling was done using Agilent and Affymetrix microarrays. Our analysis revealed distinct, non-overlapping changes in gene expression between the two doses. We found that the majority of transcriptional changes were observed at 24 hours post-injection with both doses. Interestingly, asymptomatic exposure produced more persistent transcriptional effects - in response to symptomatic dose exposure, we observed only one differentially expressed gene one week after exposure, compared to 26 in the asymptomatic dose at the same time (FDR <0.05). GO term analysis revealed that symptomatic DA exposure affected genes associated with peptidyl proline modification and retinoic acid metabolism. Asymptomatic exposure caused differential expression of genes that were associated with GO terms including circadian rhythms and visual system, and also the neuroactive ligand-receptor signaling KEGG pathway. Overall, these results suggest that transcriptional responses are specific to the DA dose and that asymptomatic exposure can cause

47 long-term changes. Further studies are needed to characterize the potential downstream
48 neurobehavioral impacts of DA exposure.

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50 **1. Introduction**

51 DA is a potent neurotoxin naturally produced by some subspecies of the marine diatom
52 *Pseudo-nitzschia*. DA accumulates in filter-feeders, including several commercially important
53 shellfish, and can cause headache, disorientation, weakness, vomiting, diarrhea, and memory loss
54 in human consumers (Perl et al., 1990). In very severe cases, exposure can lead to seizures,
55 coma, and ultimately death (Perl et al., 1990). DA exerts its neuroexcitatory effect through high-
56 affinity agonistic binding to kainate, AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic
57 acid), and NMDA (N-methyl-D-aspartic acid) ionotropic glutamate receptors (Berman and
58 Murray, 1997). This overstimulation results in excessive release of calcium ions, which can
59 cause neuronal degeneration and brain damage. The combination of neuroexcitotoxic effects
60 resulting from DA poisoning is called Amnesic Shellfish Poisoning (ASP), an illness that
61 constitutes a significant public health concern. DA was first recognized as a seafood toxin in
62 1987 when consumption of contaminated mussels on Prince Edward Island, Canada caused 153
63 cases of acute intoxication and three deaths (Perl et al., 1990). DA poisoning also occurs in
64 marine mammals and seabirds, resulting in recurring morbidity and mortality events (Bejarano et
65 al., 2008; Goldstein et al., 2008; Scholin et al., 2000; Torres De La Riva et al., 2009; Work et al.,
66 1993). In 2015, the North American west coast saw a record-breaking outbreak of *Pseudo-*
67 *nitzschia* and losses of millions of dollars in subsequent fishery closures (McCabe et al., 2016).
68 Since the first documented ASP event in 1987, seafood regulations have effectively minimized
69 human risk of acute poisoning by limiting harvest of shellfish tissue to asymptomatic

concentrations of < 20 µg DA/g tissue (Wekell et al., 2004). However, little is known about the long-term effects of chronic exposure to concentrations below regulatory limits. This knowledge gap is of particular concern for coastal and tribal communities that regularly consume shellfish containing low concentrations of DA (Ferriss et al., 2017; Lefebvre et al., 2019; Lefebvre and Robertson, 2010).

Effects of low-dose DA exposure have been demonstrated in various animal models. Recent studies in adult non-human primates (*Macaca fascicularis)* revealed that long term DA exposure at asymptomatic doses causes intentional tremors as well as changes in brain morphometry (Burbacher et al., 2019; Petroff et al., 2019). Studies in mice have demonstrated that long term low dose exposure to DA causes changes in activity and cognitive deficits (Lefebvre et al., 2017; Schwarz et al., 2014; Sobotka et al., 1996). However, the underlying gene expression changes associated with these neurobehavioral defects are not fully understood. In a previous study, we demonstrated that a single dose exposure to adult zebrafish was sufficient to impact gene expression in the brain at 6 hours post-DA injection, including the downregulation of genes involved in immune function, RNA processing, metabolism, signal transduction, and ion transport (Lefebvre et al., 2009). However, the long-term effects of a single exposure are unknown. Hence, the objectives of this study were to further investigate the long term effects of a single exposure to both symptomatic and asymptomatic DA concentrations on zebrafish brain. To this end, we conducted microarray analyses to assess brain gene expression changes after a single symptomatic and asymptomatic dose at 24, 48 and 168 hours post-exposure.

2. Materials and Methods

2.1 Experimental animals

Wild-type zebrafish (*Danio rerio*, AB strain), approximately five months of age, were obtained from Oregon State University (Sinnhuber Aquatic Research Laboratory Corvallis, OR). Fish were maintained at the Northwest Fisheries Science Center (NWFSC, Seattle, WA) in a ZebTec stand-alone recirculating and continuously-monitored zebrafish rack system with UV sterilizer (Techniplast, Exton, PA). Water temperature was maintained at 26°C, and fish were kept on a 12:12 hr light:dark cycle and fed daily with BioVita fish feed (Bio-Oregon, Longview, WA). The work was performed at the National Oceanic and Atmospheric Administration's (NOAA) Northwest Fisheries Science Center (NWFSC). This federal agency does not have an Institutional Animal Care and Use Committee (IACUC). We followed guidelines used by the University of Washington's IACUC, but all live zebrafish handling was performed at NOAA/NWFSC.

2.2 Experiment 1: Symptomatic domoic acid exposure

Adult zebrafish were exposed to domoic acid (DA) or an identical volume of vehicle (PBS) via intracoelomic (IC) injection at one year of age. DA was purchased from Sigma Chemical Corp. (St. Louis, MO). The dose of DA used in this experiment was 1.1 ± 0.2 µg /g fish. Dose standard deviations represent slight differences in individual fish body weights. Injections were of 10µL volume delivered with 33-gauge needle in a custom-made auto-injector from Hamilton® (Reno, NV). After each injection, fish were observed for 30-45 minutes to note the occurrence of any neurobehavioral excitotoxic signs (e.g., circle- or spiral-swimming). This is considered to be a "symptomatic dose" as these fish showed behavioral symptoms soon after injection, which were ameliorated within five minutes. Each treatment consisted of 10-12 individual fish (biological replicates) per time period. Zebrafish were euthanized via

decapitation and brains were dissected at 24, 48 and 168 hours post-injection as described in Lefebvre et al. (2009). In summary, whole brains were surgically removed, rinsed in ice cold PBS, and flash frozen in liquid nitrogen.

2.3 Experiment 2: Asymptomatic DA exposure

Adult zebrafish were exposed to a 0.31 ± 0.03 μg/g dose of DA or an identical volume of vehicle (PBS) via IC injection at seven months of age. Exposures and sampling were done as described in Experiment 1. This dose produced no behavioral signs of excitotoxicity and is less than the EC_{50} of DA (0.86 μg/g) in zebrafish (Lefebvre et al., 2009). Each treatment consisted of 10-12 individual fish (biological replicates) per time period. Zebrafish were euthanized via decapitation and brains were dissected at 24, 48 and 168 hours post-injection as described in Lefebvre et al. (2009).

2.4 Dose validation

All doses were validated before injection via standard high-performance liquid chromatography-ultraviolet detection (HPLC-UV) detection methods as described previously (Lefebvre et al., 2009).

2.5 Total RNA isolation

Global transcriptome analysis was conducted using microarrays. Analysis was done on three RNA replicates per time point, each consisting of RNA from 3-4 pooled brains. Total RNA was isolated from zebrafish brains using the miRNeasy Mini Kit (Qiagen Inc., Valencia, CA) following manufacturer's instructions, and stored at -70°C. RNA quantity and quality ($OD_{260/280}$ and $OD_{260/230}$ ratios) were determined using a NanoDrop ND-1000 Spectrophotometer (Thermo

Fisher Scientific, Waltham, MA). RNA integrity was characterized using the Agilent RNA 6000 Nano Kit with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Only total RNA samples with RNA integrity numbers greater than 8 were used for microarray analysis.

2.6 Microarray analyses

Microarray analysis was conducted using Affymetrix GeneChip® Zebrafish Genome Arrays (Affymetrix, Inc. Santa Clara, CA) and Zebrafish (V3) Gene Expression Microarray (Agilent Technologies, Inc. Santa Clara, CA) in Experiments 1 (symptomatic exposure) and 2 (asymptomatic exposure), respectively. Array hybridization, processing and data analysis were done as previously described (Hiolski et al., 2014; Lefebvre et al., 2009).

Data analysis was conducted using Bioconductor (Gentleman et al., 2004). Affymetrix array data were normalized using a quantile normalization procedure and the probes were summarized for each probe set using the RMA algorithm (Irizarry et al., 2003), as implemented in the Bioconductor oligo package (Carvalho and Irizarry, 2010). Probe sets were first filtered to remove all control probe sets. We then filtered out probes that did not also appear on the Agilent array used for the asymptomatic DA dose, based on the NCBI Gene ID. This resulted in 9785 genes. Since some genes are measured more than once on this array, we present the average of any duplicated genes.

Agilent microarrays were preprocessed by doing background correction using the 'normexp' function (Ritchie et al., 2007). The data were normalized using a quantile normalization (Smyth and Speed, 2003) and any genes that were not also represented in the Affymetrix platform were filtered out of the dataset. A weighted analysis of variance (ANOVA) model was fit and all comparisons made using empirical Bayes adjusted contrasts using the

Bioconductor limma package (Ritchie et al., 2015). Gene annotations were updated to the latest version of the zebrafish genome (GRCz11) by querying the probe sequences using standalone BLAST+ (Agilent arrays) or using BioMart (Affymetrix). All raw and processed data files are deposited in NCBI Gene Expression Omnibus database (GSE152814, and GSE34716 for symptomatic and asymptomatic samples, respectively).

Gene Ontology analysis (GO) of differentially expressed genes was performed for each comparison using the biological process (BP) ontology terms. We further determined the relationships between all significant GO terms by drawing directed acyclic graphs using WebGestalt (http://www.webgestalt.org/2017/GOView/). Only child terms (eg. a GO term closer to the leaf nodes of the graph than to the root) with a unique set of genes were considered.

Additionally, we conducted a cross-comparison analysis between the dose experiments to determine whether there were similarities in the GO terms and pathways enriched by each dose. Significant GO terms from the symptomatic analysis were tested in the asymptomatic dataset using a self-contained gene set test (the roast function from the Bioconductor limma package (Wu et al., 2010)) to determine whether there is evidence for significant enrichment of these pathways in the asymptomatic data. Similarly, significant GO terms from the asymptomatic analysis were tested against the symptomatic data. Pathway analysis of the differentially expressed genes (DEGs, FDR<0.05) was conducted using the Bioconductor SPIA package (Tarca et al., 2022). Significant KEGG pathways in the symptomatic set were tested for significance in the asymptomatic set and vice versa as described for the GO analysis. **2.7**

Quantitative real time PCR

Complementary DNA (cDNA) was synthesized using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA). qPCR primers were designed using Amplify software (version 4; University of Wisconsin, Madison). The primer sequences, annealing temperature, amplicon size, and amplification efficiency of each primer are provided in Table 1. qPCR was performed using the CFX96 Touch™ Real Time System (Bio-Rad, Hercules, CA) with the following protocol: 1 cycle of 95°C for 3 min; 40 cycles of 95°C for 15 sec followed by 30 seconds at primer-specific annealing temperature (65-68°C, see Table 1). Immediately following the PCR protocol, melt curve analysis was conducted to determine the quality of the amplicon (1 cycle of 65°C for 5 sec; 80 cycles of 5 sec each starting at 65°C with a 0.5°C increment at each step up to 95°C). We normalized the expression of target genes by using the mean of two house-keeping genes (*arnt2* and *beta-actin).* To account for variation in amplification efficiency (E), $E^{-\Delta Ct}$ values were calculated using the Pfaffl method, which does not assume 100% efficiency for all primer pairs (Pfaffl, 2002). Mann-Whitney tests were performed in PRISM 9.4.1 for Windows (GraphPad Software, San Diego, CA). Individual amplification efficiencies were calculated with LinRegPCR software (Ruijter et al., 2009).

3. Results

3.1 Symptomatic Exposure to DA

Symptomatic exposure of adult zebrafish to DA zebrafish caused time-dependent changes in gene expression. At 24 hours post-injection, 238 genes were differentially expressed (Fig. 1, Supplemental Fig. 1). Among them, 92 and 146 genes were up and down-regulated, respectively. In contrast, at 48 h only 35 genes were differentially expressed. Of the 35 DEGs observed at 48 h, 16 genes were upregulated and 19 were downregulated. Only one differentially expressed

gene – *oga* (Glycoside hydrolase O-GlcNAcase) - was downregulated at 168 h post-exposure. No other genes were differentially expressed at this timepoint. Comparison of the DEGs at three different time points revealed very little overlap. There were only five genes shared between the 24 h and 48 h timepoints – *hic1l* (hypermethylated in cancer 1-like), *foxg1b* (forkhead box G1b), *fkbp5* (FKBP prolyl isomerase 5), *pik3r3a* (phosphoinositide-3-kinase regulatory subunit 3a), and *si:ch211-250g4.3*.

The majority of DEGs in this analysis did not overlap those in the zebrafish brain observed more immediately (6 hours) after exposure (Lefebvre et al., 2009). For the 24 h timepoint in the symptomatic dataset, 13 genes were shared: *rbbp6* (retinoblastoma binding protein 6), *ankrd12* (ankyrin repeat domain 12), *pcdh2ac* (protocadherin 2 alpha c), *jak1* (janus kinase 1), *phf2* (PHD finger protein 2), *calm1a* (calmodulin 1a), *cadm3* (cell adhesion molecule 3), *pik3r3a* (phosphoinositide-3-kinase, regulatory subunit 3a (gamma)), *scn8a* (sodium channel, voltage gated, type VIII, alpha subunit a), *slc16a9* (solute carrier family 16 member 9a), *nipbl* (NIPBL cohesin loading factor b), *mical3* (microtubule associated monooxygenase, calponin and LIM domain containing 3a), and *foxg1b*. In the 48 h timepoint, five genes – *rgs9b* (regulator of G protein signaling 9b), *igfbp1a* (insulin-like growth factor binding protein 1a), *slc4a1* (solute carrier family 4 member 1a), *pik3r3a,* and *foxg1b* – were differentially expressed in this analysis and in Lefebvre et al., 2009, respectively. Most of these genes were primarily associated with signal transduction pathways in the latter analysis.

Functional Classification of DEGs Using GO Annotations and KEGG Pathway Analysis

Functional annotation of DEGs at 24 h and 168 h post-exposure resulted in no significant GO terms (p-value < 0.01). DEGs at 48 h post-exposure were associated with the GO terms

associated with protein modification, metabolism, and receptor signaling (Table 2). Of all significant child terms associated with symptomatic exposure at any timepoint, none were found to also be associated with asymptomatic exposure. KEGG pathway analysis revealed that DEGs at 24 h post-exposure enriched apelin signaling and oocyte meiosis pathways (Table 3).

3.2 Asymptomatic Exposure to DA

Comparison of individual time point DEGs revealed that 116 genes are differentially expressed at 24 h post-injection, 14 genes at 48 h post-injection, and 26 genes at 168 h post-injection (FDR<0.05; Fig. 1, Supplemental Fig. 1). Among the 116 DEGs at 24 h post-injection, 70 were upregulated and 46 downregulated. Whereas at 48 h, ten and four were up and downregulated, respectively. At 168 h post-injection, 11 and 15 genes were up and downregulated, respectively. Five genes were shared between the 24 and 168 h datasets – *cry5* (cryptochrome circadian regulator 5*), tcp11l2* (t-complex 11, testis-specific-like 2)*, cry-dash* (cryptochrome DASH)*, cpdp* (CPD photolyase)*,* and *bhlhe41* (basic helix-loop-helix family, member e41). Similar to the symptomatic set, there was little overlap with the asymptomatic DEGs at 6 h in Lefebvre 2009. Only two genes were shared between 6 h and 24 h – *tob1b* (transducer of ERBB2, 1b) and *tcp11l2* – and two between 6 h and 168 h - *tcp11l2* and *nptx1l* (neuronal pentraxin 1 like). No genes were shared at the 48 h timepoint.

Functional Classification of DEGs Using GO Annotations and KEGG Pathway Analysis

Functional annotation showed that the genes differentially expressed at 24 h are associated with GO terms associated with circadian rhythms, DNA modification, regulation of transcription, cell fate, and mitochondrial respiration (Table 4). At 48 h post-injection, enriched GO terms were associated with light sensing and phototransduction (Table 4). At 168 h post-

injection, DEGs associated with GO terms including circadian rhythms, response to environmental insults, and transcription (Table 4). DEGs at 24 hours enriched the KEGG pathways cell cycle, protein processing in endoplasmic reticulum, neuroactive ligand-receptor interaction, and apoptosis (Table 5). At 48 and 168 hours, they enriched phototransduction and cytosolic DNA-sensing, respectively (Table 5).

3.3 Comparison between Symptomatic and Asymptomatic Datasets

There was very little overlap between the asymptomatic and symptomatic gene sets. Only two genes were differentially expressed (FDR < 0.05) in both the 24 h asymptomatic and symptomatic datasets – *vipr2* (vasoactive intestinal peptide receptor 2) and *or115-1* (odorant receptor, family F, subfamily 115, member 1) – and none in the 48 h or 168 h datasets. Our cross-comparison analysis also revealed large differences in the GO terms and pathways enriched by symptomatic and asymptomatic exposure. In our self-contained gene set test using the significant GO and KEGG terms from the symptomatic analysis, we found evidence of significant enrichment of the Apelin signaling pathway in the asymptomatic dataset (Table 3). In the reverse test using the GO terms from the asymptomatic analysis, mitochondrial respiratory chain complex assembly, regulation of gastrulation, negative regulation of catalytic activity, and DNA repair were all significantly enriched in the symptomatic dataset at 24 hours. None of the asymptomatic GO terms were also found to be enriched in the symptomatic dataset at 48 and 168 hours.

3.4 Quantitative PCR Validation

We validated microarray results for seven randomly selected genes – *calm3a* (calmodulin 3), *srrt* (serrate RNA effector molecule homolog), *gale* (UDP-galactose-4-epimerase), and

pias4a (protein inhibitor of activated STAT 4a)*, neil* (nei-like DNA glycosylase 1)*, cry1bb* (cryptochrome 3b)*,* and *cpd-phr* (CPD photolyase) - using quantitative reverse transcriptase (qRT)-PCR. Amplification efficiency of the primers varied between 1.725-2.114 (Table 1). At the 24 hr post-injection time point, genes *srrt*, *gale, neil1*, and *cpd-phr* were upregulated in response to DA exposure, confirming microarray results (Fig. 2, Supplemental Table 1). Additionally, *pias4a* was not significantly differentially regulated, also concurring with microarray results. At 48 hours, genes *calm3a, neil1, cry1bb,* and *cpd-phr* agreed with microarray results, showing no significant differential expression. At 168 hours, four out of the seven genes (*calm3a, srrt, gale,* and *pias4a)* concurred with microarray results. Disagreement with our microarray results may be due to various technical factors previously identified (Freeman et al., 1999; Morey et al., 2006; Yang et al., 2002).

4. Discussion

4.1 Gene expression changes resulting from asymptomatic exposure are distinct from and appear to persist longer than those from symptomatic exposure

One major finding from this analysis is that symptomatic and asymptomatic doses produced functionally distinct changes in gene expression, consistent with previous studies (Hiolski et al., 2014; Lefebvre et al., 2009). For both doses, there was very little overlap in the differentially expressed genes, GO terms, and KEGG pathways that were enriched across time points, which corroborates previous studies reporting large time-dependent differences in the gene expression profiles of zebrafish exposed to DA (Hiolski et al., 2014; Lefebvre et al., 2009). Interestingly, we found more DEGs at 168 hours in the asymptomatic set than in the symptomatic set, suggesting that the effects of asymptomatic DA exposure may persist longer

than those of symptomatic exposure. The observed attenuation of gene expression changes by 168 h in the symptomatic set suggests that, while this dose produces behavior associated with acute neurotoxicity, individuals may be able to recover following acute exposure without long-term changes to the transcriptome.

4.2 Both asymptomatic and symptomatic DA exposure alter genes involved in mitochondrial function

Both doses showed differential expression of genes associated with mitochondrial function at 24 h post-exposure, though these genes were not the same between doses. DA-induced mitochondrial dysfunction has been previously demonstrated in mice (Lu et al., 2012) and zebrafish (Hiolski et al., 2014). Acute high-dose DA exposure causes neuron death by excess influx of Ca^{2+}, which can be taken up by mitochondria through *mcu* (mitochondrial calcium uniporter) (De Stefani et al., 2011). At 24 h, *mcu* was upregulated in the symptomatic dataset. Mitochondrial uptake of Ca^{2+} modulates increases in cytosolic Ca^{2+} following glutamate insults (Wang and Thayer, 1996), however, this uptake has also been shown to drive neuronal death (Schinder et al., 1996; Stout et al., 1998). At the same timepoint, the symptomatic dose downregulated genes encoding mitochondrial ribosome proteins (*mrpl1, mrpl30, mrpl47,* and *mrpl52*). Effects on mitochondrial ribosomal genes may alter protein synthesis, particularly of proteins involved in energy conversion and ATP production (Greber and Ban, 2016). One previous study has found that chronic low-level DA exposure resulted in a reduction of maximum mitochondrial respiration rates in zebrafish, as well as a compensatory increase in mitochondrial biogenesis (Hiolski et al., 2014). Additionally, in our asymptomatic dataset, we found differential expression of genes in mitochondrial respiratory chain complex assembly - *sdhaf3* (succinate dehydrogenase complex assembly factor 3)*, cox17* (cytochrome c oxidase

copper chaperone), and *ndufa4* (NDUFA4 mitochondrial complex associated) - at 24 h, which may reflect changes in ATP production. These findings suggest that mitochondria may be a target of DA excitotoxicity at both asymptomatic and symptomatic doses.

4.3 Symptomatic DA exposure may affect CNS through post-translational modifications and changes to retinoic acid metabolism

DA exposure has been demonstrated to cause cognitive deficits in rodents (Kuhlmann and Guilarte, 1997; Lefebvre et al., 2017; Petrie et al., 1992) and humans (Grattan et al., 2018, 2016), which have been attributed to acute hippocampal neuron loss. Our results suggest that several pathways critical for neuronal health are enriched following symptomatic DA exposure, including apelin signaling. Apelin signaling regulates angiogenesis, vasodilation, and constriction via the activation of calmodulins (Helker et al., 2020), two of which - *calm3b* and *calm1a* are differentially expressed in this study (Chapman et al., 2014). Furthermore, certain apelins are neuroprotective of cortical neurons by alleviating the increase in intracellular Ca^{2+} caused by NMDA-mediated neurotoxicity (Cheng et al., 2012) and by modulating NMDAR activity (Cook et al., 2011).

At 48 h, we observed enrichment of the GO term peptidyl proline modification, a type of non-covalent post-translational modification that can affect neuron health and function. Among the genes that enriched this GO term was *egln3* (egl-9 family hypoxia-inducible factor 3), which encodes an enzyme that regulates neuronal apoptosis and was downregulated in this analysis (Lee et al., 2005). *fkbp5* (FKBP prolyl isomerase 5) - a glucocorticoid receptor chaperone that plays a role in the stress response and is a risk factor for a number of psychiatric diseases (Zannas et al., 2016) - was also downregulated in our analysis, consistent with previous analysis

of zebrafish brains chronically exposed to DA (Hiolski et al., 2014). Overexpression of FKBP5 protein in mice impairs spatial learning by altering AMPA receptor recycling and glutamatergic transmission (Blair et al., 2019). Previous studies have identified DA-induced changes to long term potentiation (LTP) (Qiu et al., 2009), a synaptic mechanism mediated by AMPARs and that underlies learning. These changes in post-translational modification represent one potential avenue by which DA may alter neuronal health, learning, and memory.

Symptomatic DA exposure at 48 h may also cause changes in retinoic acid metabolism. We observed downregulation of *cyp26a1* (cytochrome P450 family 26 subfamily A member 1, a retinoic acid metabolizing enzyme) and other genes. *cyp26a1* maintains proper signaling during CNS development by attenuating retinoic acid (RA) expression (Emoto et al., 2005; Rydeen et al., 2015). *Dhrs3a* (dehydrogenase/reductase (SDR family) member 3a), another gene that regulates RA biosynthesis in the CNS (Feng et al., 2010), was also downregulated. Disruption of retinoid signaling during development has been shown to have persistent behavioral effects, including decreased social affiliation in adult zebrafish (Bailey et al., 2016). Studies in other models suggest that in adulthood, RA is involved in neuron differentiation, regeneration, and synaptic plasticity, and that abnormal RA levels may contribute to nervous system dysfunction (Maden, 2007; Mey and McCaffery, 2004).

4.4 Asymptomatic DA exposure results in changes to circadian rhythm, the visual system, and various neuroactive ligand signaling pathways.

Circadian rhythms entrain the fluctuation of various physiological processes with various environmental cues, most notably light. Disruptions in rhythmicity are associated with cognitive

dysfunction, and recent studies have shown circadian rhythms regulate synaptic plasticity and memory formation (Hartsock and Spencer, 2020; Krishnan and Lyons, 2015). We found enrichment of the GO term "circadian regulation of gene expression" at 24 and 168 h following exposure. Glutamate is the primary neurotransmitter involved in transmitting light signals to the superchiasmatic nucleus, which acts as a central pacemaker to regulate circadian rhythms in mammals (Castel et al., 1993; Ding et al., 1994). Since DA binds with high-affinity to glutamate receptors, it is possible that exposure could disrupt downstream processes that are dependent on glutamatergic signaling, including circadian rhythm regulation. Zebrafish *per2* (period circadian clock 2) and *cry1a* (cryptochrome circadian regulator 1a) - two genes that were downregulated at 168 h in our analysis - help establish rhythmic gene expression (Tamai et al., 2007; Wang et al., 2015). Knockdown of *cry1a* and *per2* has been demonstrated to cause defective synchronization of cellular clocks, disruption of behavioral rhythms, and impaired cellular metabolism in zebrafish larvae (Hirayama et al., 2019).

In addition, we observed differential expression of genes associated with multiple neuroactive ligand-receptor interactions, including *sst1.1* (somatostatin1.1), *vipr2* (vasoactive intestinal peptide receptor 2) and *p2rx4a* (purinergic receptor P2X, ligand-gated ion channel, 4a). All these genes have been implicated in the modulation of various CNS functions, including immune regulation (Patel, 1999; Suurväli et al., 2017; Waschek, 2013). DA has been shown to induce microglial activation in *in vitro* culture and in rat brain (Ananth et al., 2001; Mayer et al., 2001) and induce antibody response in humans, zebrafish, and sea lions. (Lefebvre et al., 2019, 2012). In California sea lions diagnosed with domoic acid toxicosis, upregulation of the inflammatory cytokine *tnfa* (tumor necrosis factor alpha) has been observed (Mancia et al.,

2012). Given the critical role of the neuroimmune system in health, these findings highlight the need for further studies on the immune system as a potential target of DA.

Additionally, both KEGG and GO term analyses suggest modifications to the visual system 48 hours post-exposure. Multiple genes involved in the GO term visual perception were upregulated, including two opsins (*opn1lw1* and *opn1lw2*) and two photoreceptor phosphodiesterases (*pde6ha* and *pde6a*). In zebrafish, visual sensitivity and expression of opsin and phosphodiesterase genes vary with circadian oscillations (Abalo et al., 2020). One question this analysis raises is whether DA-induced changes in circadian rhythm might lead to downstream effects on visual acuity or vice versa, and if so, what practical effects this might have on behavior. Many zebrafish behaviors are reliant on visual perception, including prey capture, predator avoidance, and visual startle response (Neuhauss, 2003). Further studies should take advantage of the many behavioral assays available in the zebrafish model to assess the effect of DA on these endpoints (Neuhauss, 2003).

4.5 Relevance to Human Exposures

Consumption of DA at concentrations below regulatory limits occurs in certain human populations and is associated with memory deficits (Grattan et al., 2018, 2016). Understanding the risks associated with low-dose exposure is critical for establishing consumption guidelines and regulations that protect from adverse health effects. We demonstrate that, surprisingly, asymptomatic exposure produces transcriptomic changes that persist longer and are distinct from symptomatic exposure. It further identifies potential mechanisms that could underly observed cognitive deficits associated with low-dose exposure – namely effects on neuroactive ligand signaling in the immune system and circadian rhythms. These processes are critical for proper

CNS function and further research should be conducted to understand their role in mediating DA toxicity.

5. Conclusions

Our analyses demonstrate that a single exposure to DA at symptomatic and asymptomatic doses produce unique transcriptional changes. While transcriptional changes were attenuated by one week in the symptomatic set, asymptomatic exposure caused significant gene expression changes through the one-week time point, suggesting that asymptomatic exposure to DA may result in more persistent effects. Our findings emphasize the need for further studies on asymptomatic exposure to determine its unique health impacts. Additional investigation on these transcriptional effects of acute DA exposure at both doses are required to determine downstream neurological impacts. One limitation of this study is the use of two different microarray platforms for the two analyses (Affymetrix for the symptomatic exposure and Agilent for the asymptomatic). To address this, we restricted our analysis to only the genes that were common to the two platforms, and used a gene set test for the comparison analysis.

6. Acknowledgements

Support for this research was provided by NIEHS P30 ES007033, an NSF GRFP (112374), NIH R01s ES021930 and ES030319 (to KL and David Marcinek University of Washington), NSF R01s OCE-1314088 and OCE-1839041 (to KL and David Marcinek University of Washington), and an Internal Grant from NOAA Northwest Fisheries Science

Center (to KL). Additional support was provided by the Woods Hole Center for Oceans and Human Health (NIH P01ES028938 and NSF 1840381), and we would like to thank Dr. John Stegeman and Dr. Mark Hahn for their work in obtaining this funding.

7. CRediT Author Statement

Alia Hidayat: Validation, Formal analysis, Writing – Original draft preparation, Visualization. **Kathi A. Lefebvre:** Conceptualization, Methodology, Investigation, Resources, Writing – Review and Editing, Project administration, Funding acquisition. **James MacDonald:** Formal analysis, Writing – Review and Editing, Data curation. **Theo Bammler:** Formal analysis, Writing – Review and Editing, Data curation. **Neelakanteswar Aluru:** Supervision, Writing – Review and Editing.

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678 **List of Tables**

679 **Table 1. qPCR Primers Used in This Study**. Primer sequences (5'-3'), amplicon size (base pairs, bp), annealing temperature (Tm)
680 and primer (amplification) efficiency of target and housekeeping genes are provided.

Gene	Forward Primer	Reverse Primer	Amplicon size (bp)	T_m (°C)	Primer Efficiency
calm3a	TTCCCGAGTTCTTGACGATG	TAGCCATTGCCGTCCTTATC	103	65	2.11
srrt	CGGAGTCGGTCAAACGCTACAA	CTTTGAGCGAAACCACTCTTCATCT	108	65	2.03
gale	GCTTCAGGTCGAAAGATTGCATATC	CTTCCAACCCAGCTCTTTCTC	104	65	1.88
pias4a	GGAGGCCAATCAGAGATGATAAAG	CGCCTCAGGAACACATATATCC	95	67	1.75
cry1bb	GGAGGAGGGCATGAAGGTGTT	ACAGTAACAGTGGAAGAACTGCTGG	120	68	1.85
neil1	ACTCCACACAGGGTTCTGAGCTT	TTAGAAAGAACATTCTCCCTGAAGC	133	67	1.75
cpd-phr	GGTAAAGGAATGCAAAAGCCTG	GGAGATTCTAAGAGGGTTGAAATCG	132	67	1.75
arnt2	ATCGCAACACTGCTTTCGATGTG	CAGCCTGCTGACTGTGATGTTGAC	117	67	1.88
***β**-actin*	CAACAGAGAGAAGATGACACAGAT CA	GTCACACCATCACCAGAGTCCATCAC	140	65	1.73

681

682

683 **Table 2: GO Term Enrichment of Symptomatic DEGs at 48 hours Post-Exposure.** GO terms found to be significantly enriched
684 (p-value < 0.01) at 48 hours post-exposure to a symptomatic dose of DA. Only significant child terms are included. No GO terms were
685 significantly enriched at the 24 and 168 h timepoints.

686

GO Term	GO ID	P-value	FDR	DEGs in GO term
Peptidyl-proline modification	GO:0018208	<0.001	0.046	*Egln3, p4ha1b, fkbp5*
Protein hydroxylation	GO:0018126	0.001	0.150	*Egln3, p4ha1b*
Vitamin metabolic process	GO:0006766	0.003	0.150	*Cyp26a1, mmadhcb*
Retinoid metabolic process	GO:0001523	0.003	0.150	*Dhrs3a, cyp26a1*
Cellular hormone metabolic process	GO:0034754	0.004	0.150	
Intracellular receptor signaling pathway	GO:0030522	0.009	0.150	
Monocarboxylic acid catabolic process	GO:0072329	0.010	0.150	*cyp26a1, hadhb*

687

688

Table 3: KEGG Pathway Analysis of Symptomatic DEGs at 24 hours Post-Exposure. KEGG pathways found to be significantly enriched (p-value < 0.1) at 24 hours post-exposure to an asymptomatic dose of DA. KEGG pathway analysis was conducted using the Bioconductor SPIA package. Asterisks indicate pathways that were also found to be significantly enriched among asymptomatic data. No KEGG pathways were found to be significantly enriched at the 48 and 168 h timepoints.

KEGG Pathway	Pathway ID	P-value	DEGs in pathway
Apelin signaling pathway*	04371	0.046	*Prkcea, calm3b, calm1a, gnai2a, pik3c3, notch3*
Oocyte meiosis	04114	0.057	*Calm3b, rps6kal, calm1a, pkmyt1, ppp2r1bb*

698 **Table 4: GO Term Enrichment of Asymptomatic DEGs.** GO terms found to be significantly enriched (p-value < 0.01) at 24, 48, or
699 168 hours post-exposure to an asymptomatic dose of DA. Asterisks indicate GO terms that were additionally found to be significantly
700 enriched among asymptomatic data.

Time point (h)	GO Term	GO ID	P-value	DEGs in GO term
24	Circadian regulation of gene expression	GO:0032922	0.001	*Ciarta, bhlhe41, cry4, cry3a*
	DNA alkylation	GO:0006305	0.001	*Hells, dnmt3ba, gp9*
	DNA methylation or demethylation	GO:0044728	0.001	
	Mitochondrial respiratory chain complex assembly*	GO:0033108	0.003	*Sdhaf3, cox17, ndufa4*
	Regulation of cell fate specification	GO:0042659	0.002	*Eve1, gp9, ace*
	Cell fate commitment involved in formation of primary germ layer	GO:0060795	0.005	
	Regulation of gastrulation*	GO:0010470	0.006	
	Negative regulation of catalytic activity*	GO:0043086	0.006	*Timp2a, spry2, sh3bp51a, mcl1a, mcm2*
	Entrainment of circadian clock by photoperiod	GO:0043153	0.006	*Cry5, cry3a*
	Negative regulation of transcription, DNA-templated	GO:0045892	0.009	*ciarta, bhlhe41, hes6, cry3a, atf4a, phc1, tbx18, sox9a, smad4a*

	Glycerolipid catabolic process	GO:0046503	0.009	*Lipg, pla2g4aa*
	Regionalization	GO:0003002	0.009	*bhlhe41, hes6, eve1, foxa3, tbx18, gp9, spry2, ace, smad4a*
	DNA repair*	GO:0006281	0.010	*Cpdp, cry5, cry-dash, xrcc1, xpc, mcm2, mms19*
48 h	Visual perception	GO:0007601	<0.001	*opn1lw1, pde6ha, opn1lw2, pde6a, cnga1a*
	Protein-chromophore linkage	GO:0018298	<0.001	*opn1lw1, opn1lw2*
	Phototransduction	GO:0007602	<0.001	
	Cellular response to light stimulus	GO:0071482	<0.001	
	G protein-coupled receptor signaling pathway	GO:0007186	0.003	*opn1lw1, gngt2a, opn1lw2*
168 h	Circadian regulation of gene expression	GO:0032922	<0.001	*Cry1a, bhlhe41, per2, cry5*
	Entrainment of circadian clock by photoperiod	GO:0043153	<0.001	*Cry1a, per2, cry5*
	Response to cold	GO:0009409	0.001	*bhlhe41, per2*
	Response to hydrogen peroxide	GO:0042542	0.001	*Cry1a, per2*
	Response to UV	GO:0009411	0.001	*Ddb2, per2*

	Negative regulation of transcription, DNA-templated	GO:0045892	0.001	*Cry1a, arntl1a, bmp2b, bhlhe41, per2*
	Cardiac muscle cell development	GO:0055013	0.001	*Bmp2b, sept15*
	Bicellular tight junction assembly	GO:0070830	0.002	*Cldni, cldn10l2*
	DNA repair	GO:0006281	0.002	*Ddb2, cry-dash, cpdp, cry5*

701

702

703 **Table 5: KEGG Pathway Analysis of Asymptomatic DEGs.** KEGG pathways found to be significantly enriched (p-value < 0.1) by
704 differentially expressed genes at 24, 48, and 168 hours post-exposure to an asymptomatic dose of DA. KEGG pathway analysis was
705 conducted using the Bioconductor SPIA package.

Timepoint	KEGG Pathway	PathwayID	P-Value	DEGs in pathway
24 h	Cell cycle	04110	0.045	*Smad4a, mad2l1, mcm2*
	Protein processing in endoplasmic reticulum	04141	0.053	*rrbp1a, dnajc3a, atf4a, man1a1*
	Neuroactive ligand-receptor interaction	04080	0.089	*sst1.1, vipr2, p2rx4a*
	Apoptosis	04210	0.094	*map2k2a, mcl1a, atf4a*
48 h	Phototransduction	04744	<0.001	*Opn1mw2, pde6a, cnga1a*
168 h	Cytosolic DNA-sensing pathway	04623	0.093	*irf7*

706

List of Figures

Figure 1.

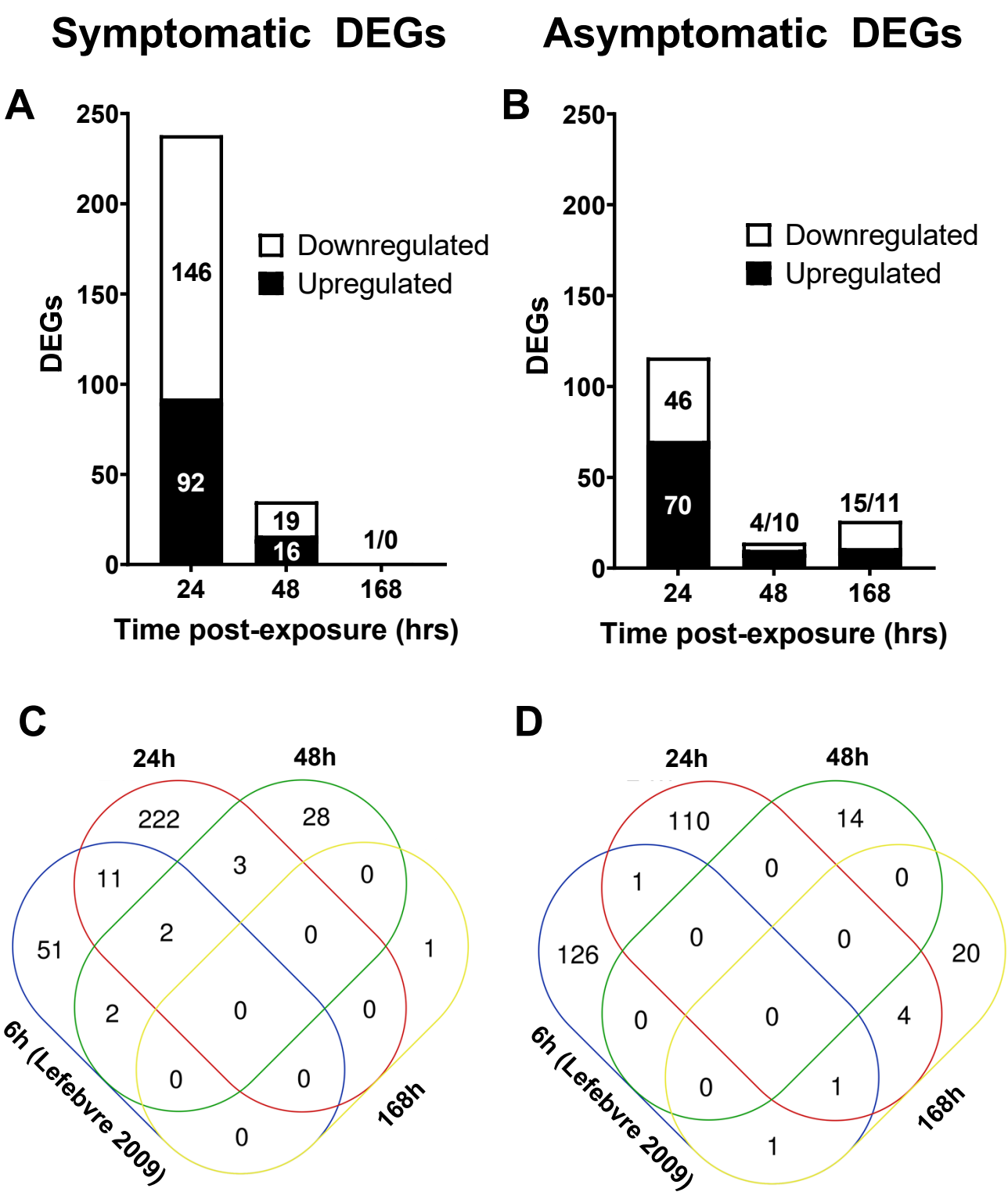
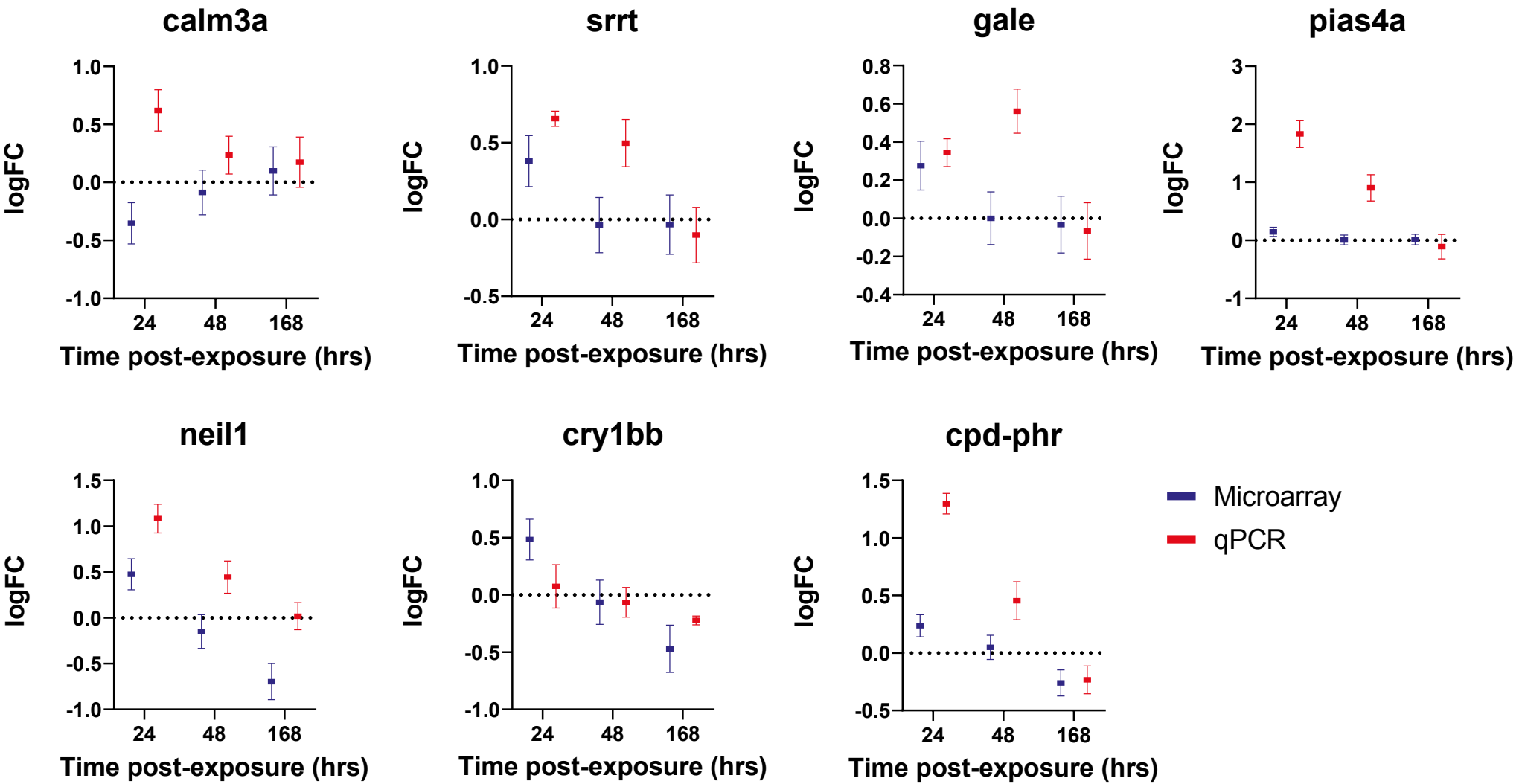

Symptomatic DEGs
Asymptomatic DEGs
A
B
DEGs
Downregulated
Upregulated
146
92
19
16
1/0
46
70
4/10
15/11
24
48
168
Time post-exposure (hrs)
C
D
24h
48h
6h (Lefebvre 2009)
168h
222
28
11
3
0
51
2
0
1
2
0
0
0
0
0
110
14
1
0
0
126
0
0
20
0
0
4
0
1
1

Figure 2.


calm3a
srrt
gale
pias4a
neil1
cry1bb
cpd-phr
logFC
Time post-exposure (hrs)
24
48
168
Microarray
qPCR

Symptomatic and asymptomatic domoic acid exposure in zebrafish (*Danio rerio*) revealed distinct non-overlapping gene expression patterns in the brain – Supplementary Information

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Key words: Domoic acid; harmful algal bloom toxin; glutamate; excitotoxin

24 hours						
	Microarray			qPCR		
Gene	logFC	Standard error	FDR	logFC	Standard error	p-value
calm3a	-0.353	0.178	0.056	0.620	0.178	**0.050**
*srrt**	0.380	0.166	**0.015**	0.656	0.049	**0.003**
*gale**	0.276	0.128	**0.026**	0.344	0.073	**0.050**
*pias4a***	0.145	0.079	0.090	1.833	0.235	0.054
*neil1**	0.475	0.169	**0.002**	1.083	0.157	**0.003**
cry1bb	0.483	0.178	**0.002**	0.075	0.189	0.921
*cpd-phr**	0.237	0.096	**0.007**	1.298	0.089	**0.003**

48 hours						
	Microarray			qPCR		
Gene	logFC	Standard error	FDR	logFC	Standard error	p-value
*calm3a***	-0.087	0.193	0.999	0.234	0.163	0.485
srrt	-0.037	0.180	0.999	0.498	0.154	**0.028**
gale	0.000	0.138	0.999	0.561	0.116	**0.050**
pias4a	0.005	0.085	0.999	0.903	0.227	**0.015**
*neil1***	-0.150	0.184	0.999	0.444	0.176	0.094
*cry1bb***	-0.064	0.193	0.999	-0.064	0.129	0.628
*cpd-phr***	0.049	0.105	0.999	0.454	0.165	0.105

168 hours						
	Microarray			qPCR		
Gene	logFC	Standard error	FDR	logFC	Standard error	p-value
*calm3a***	0.099	0.207	0.990	0.174	0.217	0.802
*srrt***	-0.034	0.193	0.990	-0.102	0.181	0.867
*gale***	-0.033	0.149	0.990	-0.066	0.148	0.779
*pias4a***	0.012	0.092	0.990	-0.110	0.211	0.463
neil1	-0.697	0.197	**0.000**	0.018	0.148	0.867
cry1bb	-0.471	0.207	0.055	-0.223	0.038	**0.043**
cpd-phr	-0.261	0.113	**0.044**	-0.234	0.121	0.072

Supplemental Table 1. qPCR Results

Expression levels and statistical parameters for seven genes chosen for qPCR validation over 24, 48, and 168 hours. Statistically significant results (FDR or p-value <.05) are bolded. Asterisks represent cases where qPCR data shows statistically significant differential expression in the same fold change direction as microarray data. Double asterisks represent cases where both microarray and qPCR data do not show significant differential expression.

List of Supplementary Figures

Supplementary Figure 1. Heatmaps of differentially expressed genes (DEGs) in adult zebrafish brain at 24, 48, and 1-week time points following exposure to A) a symptomatic dose (FDR <0.05) or B) an asymptomatic dose of DA (FDR <0.05). Red and blue bars represent upregulated or downregulated DEGs, respectively.

Supplementary Figure 1.