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Proteome changes in larval zebrafish (*Danio rerio*) and fathead minnow (*Pimephales promelas*) exposed to (±) anatoxin-a

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ABSTRACT

Anatoxin-a and its analogues are potent neurotoxins produced by several genera of cyanobacteria. Due in part to its high toxicity and potential presence in drinking water, these toxins pose threats to public health, companion animals and the environment. It primarily exerts toxicity as a cholinergic agonist, with high affinity at neuromuscular junctions, but molecular mechanisms by which it elicits toxicological responses are not fully understood. To advance understanding of this cyanobacteria, proteomic characterization (DIA shotgun proteomics) of two common fish models (zebrafish and fathead minnow) was performed following (±) anatoxin-a exposure. Specifically, proteome changes were identified and quantified in larval fish exposed for 96 h (0.01–3 mg/L (±) anatoxin-a and caffeine (a methodological positive control) with environmentally relevant treatment levels examined based on environmental exposure distributions of surface water data. Proteomic concentration - response relationships revealed 48 and 29 proteins with concentration - response relationships curves for zebrafish and fathead minnow, respectively. In contrast, the highest number of differentially expressed proteins (DEPs) varied between zebrafish (n = 145) and fathead minnow (n = 300), with only fatheads displaying DEPs at all treatment levels. For both species, genes associated with reproduction were significantly downregulated, with pathways analysis that broadly clustered genes into groups associated with DNA repair mechanisms. Importantly, significant differences in proteome response between the species was also observed, consistent with prior observations of differences in response using both behavioral assays and gene expression, adding further support to model specific differences in organismal sensitivity and/or response. When DEPs were read across from humans to zebrafish, disease ontology enrichment identified diseases associated with cognition and muscle weakness consistent with the prior literature. Our observations highlight limited knowledge of how (±) anatoxin-a, a commonly used synthetic racemate surrogate, elicits responses at a molecular level and advances its toxicological understanding.

1. Introduction

Cyanobacteria thrive in diverse habitats across a wide range of environmental conditions (including polar systems, hot springs, alkaline lakes, deserts) with several species known to create secondary metabolites such as cyanotoxins. Whereas progress has been made for detection of cyanotoxins (Cevallos-Cedeño et al., 2022; Osswald et al., 2007b; Stauffer et al., 2019), as well as in ecology and biogeography associated

Abbreviations: ANTX-a, anatoxin-a; RHW, reconstituted hard water); ECD, Organization for Economic Co-operation and Development; EPA, Environmental Protection Agency; CAS, Chemical Abstracts Service; hpf, hours post fertilization; LC-MS/MS, liquid chromatography tandem mass spectrometry; FET, fish embryo toxicity; DEP, differentially expressed proteins; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

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with production of these toxins (Anderson, 2017; Granéli and Flynn, 2006), significant gaps in knowledge remain, specifically in characterization of the accompanying molecular modes and mechanisms of action in organisms. Eutrophication and warming of aquatic environments have likely led to increased frequency and intensity of cyanobacterial blooms and associated cyanotoxins (Anderson, 2017; McAllister et al., 2016) with concurrent direct and indirect impacts to uses of freshwater ecosystems, coastal resources and human health (Kudela et al., 2015). These impacts can include those directly derived from consumption of contaminated seafood and recreational exposures leading to health impacts, decreased water quality, chronic health issues and mortality, in addition to indirect impacts to water production, tourism, coastal aesthetics, livestock, and companion animals. In fact, toxins produce during harmful algal blooms (HABs) can represent the greatest water quality threats to public health and the environment in some locations at some times (Brooks et al., 2017, 2016). Despite aquatic organisms being directly exposed to cyanotoxins in the field, relatively few investigations have focused on understanding the impact of these toxins in fish. Toxicity to fish following cyanotoxin exposures in addition to their bioaccumulation were recently summarized (Banerjee et al., 2021; Ferrão-Filho and Kozlowsky-Suzuki, 2011), with publications heavily focused on microcystins compared to other understudied toxins (Merel et al., 2013).

One of the first cyanobacterial toxins to be isolated and structurally analyzed (Devlin et al., 1977), anatoxin-a (ANTX-a) ((2-acetyl-9-azabicyclo[4.2.1]non-2-ene) is a potent neurotoxin produced worldwide that is toxic to a range of species, inducing quick lethality in mammals, which led to an alternative designation of 'VFDF' (Very Fast Death Factor). Indeed, surface water contamination from this cyanotoxin and its analogues present a risk to public health and the environment (Lovin and Brooks, 2020), with anatoxins found in 59–67 % of water samples sampled in New Zealand (McAllister et al., 2016) and California, USA (Bouma-Gregson et al., 2018), respectively. Physiological and molecular targets of ANTX-a were recently reviewed (Colas et al., 2021) and highlighted the limited knowledge on its putative chronic toxicity. Further work is needed to understand interspecies differences (aquatic and terrestrial), the enantioselective toxicity of this compound, and subsequent individual and population risks for this water quality threat (Lovin et al., 2021). While implicated in the death of fish and various other animals, the mechanism of action it not fully known, especially in fish, receiving less attention than other cyanobacterial toxins such as microcystin or saxitoxin (Merel et al., 2013) despite being one of the most frequently detected cyanotoxins in freshwater (Bouma-Gregson et al., 2018; Quiblier et al., 2013). Only one enantiomer (+) ANTX-a has been found in nature or cyanobacterial cultures, while the synthetic racemate (±) ANTX-a has been commonly used in much of the toxicology literature, apparently due to practical concerns such as availability and cost (Lovin et al., 2021).

Whereas commercially synthesized (+) and (±) ANTX-a standards in addition to crude extracts of species specific cultures have played a central role in developing knowledge and understanding of ANTX-a specificity and potency as an nicotinic acetylcholine receptor (nAChR) agonist (Aráoz et al., 2005; Swanson et al., 1989; Wonnacott and Gallagher, 2006), a more complete understanding of the impact associated with ANTX-a is lacking. In fish, this information is particularly poor, with little consensus on function and toxicological/neurotoxicological response (Kaminski et al., 2013). This may be due in part to the considerable variation in the literature on the form of ANTX-a that organisms have been exposed, ranging from cultured cyanobacterial cells and purified cellular extracts to synthesized compounds. For example, while acute toxic effects were not observed in mussels exposed to a stimulated toxic bloom over 15 days (Osswald et al., 2008), nor in mice exposed to repeated sublethal levels of (+) ANTX-a for 28 days (Fawell et al., 1999), acute mortality was observed in both embryonic and juvenile carp from 24 to 96h (Osswald et al., 2009, 2007a), with altered heart rates reported in zebrafish (Oberemm et al., 1999). As prevalence and effect of cyanobacterial blooms producing ANTX-a are highly dynamic, understanding the toxicity of this cyanotoxin to aquatic and terrestrial wildlife and human health is critical, especially considering its deleterious effects on the nervous system (Colas et al., 2021).

New evidence at the molecular level may contribute to enhancing information with respect to toxicity at environmentally relevant concentrations. One way to gain an integrated view of a response pathway to a HAB toxin is to use global "omics" methods. Characterizing the identity and relative abundance of proteins in tissues or organisms is a logical first step in understanding normal biological responses to external stressors and subsequent differences in the associated response. As such, proteome profiling has proved to be a pragmatic means of discovering novel information, identifying new avenues of research and has been applied in enhancing knowledge of pathways associated with harmful algal bloom (HAB) toxicity in a number of organisms and cyanobacterial species that produce toxins (Carneiro et al., 2015; D'Agostino et al., 2014; Imanishi and Harada, 2004, 2004; Rai et al., 2018; Tonietto et al., 2012; Welten et al., 2020).

In the current study, following an initial effort to explore variation in behavior and gene expression responses to exposure of (±) ANTX-a in two common fish models (Lovin et al., 2021), proteome characterization was applied to samples collected during this experiment in common alternative vertebrate models. The combined use of these two model organisms has been previously beneficial in identifying mechanisms and pathways associated with diverse biological activities for chemicals/pharmaceuticals lacking mechanistic data (Steele et al., 2018a). This will be particularly beneficial in the study of ANTX-a toxicity where differences in sensitivity among organisms have significant implications for environmental risk evaluation following a HAB event, and further to support future studies with the naturally occurring (+) enantiomer. Existing literature studying changes in proteome as a function of cyanotoxin exposure predominantly uses MALDI-TOF, or two-dimensional gel electrophoresis (2-DE) proteomic approaches (data dependent acquisition [DDA]), which have been reported previously as time-consuming with low sensitivity and results in limited identification of proteins (100s) (Sanchez et al., 2011). Data independent acquisition (DIA) has been reported as superior to DDA approaches in quantification, reproducibility, specificity and accuracy, in addition to particularly appropriate for quantification of low abundance peptides/proteins (Barkovits et al., 2020; Tsou et al., 2016). Additionally, higher numbers of protein identifications have been observed using DIA approaches (> 2700–8000) in larval zebrafish (van der Plas-Duivesteijn et al., 2014; Langan and Brooks, 2022), making it an ideal method for characterization purposes. Therefore, to enhance our understanding of species-specific sensitivity, a DIA approach was undertaken here to characterize differential protein expression profiles in both species.

2. Materials and methods

2.1. Animal husbandry

Tropical 5D wild type zebrafish (*Danio rerio*) were maintained at Baylor University following standard culturing conditions described previously (Corrales et al., 2017; Steele et al., 2018a, 2018b). Zebrafish were kept in a Z-mod recirculating system (Marine Biotech Systems, Beverly MA, USA) at a density of <4 fish per liter. Temperature was held at 28 °C, with a pH at 7.0 ± 0.1, and salinity of 260 ppm (Instant Ocean). They were fed twice daily with artemia (Artemia sp. nauplii; Pentair AES) and flake food (Pentair AES, Apopka FL, USA) with a 16:8h light: dark photoperiod. Fathead minnow larvae were acquired (< 48h post-hatch) from a commercial laboratory (Environmental Consulting and Testing, Superior WI, USA). Culture conditions were maintained throughout the experiment at 25 °C ± 1 °C, with pH varying between 7.8 and 8.1. All experimental procedures and fish culturing protocols followed Institutional Animal Care and Use Committee protocols approved at Baylor University.

2.2. Experimental design

The experimental design employed here has been previously reported in Lovin et al., (2021), which identified species specific differences in photolocomotor response and gene expression changes. In the current study, we focused on proteome changes as a result of this exposure carried out on protein simultaneously isolated during the RNA extraction process. While most proteomic studies using larval fish do not justify the use of the number of individuals that are pooled prior to analysis, Langan and Brooks (2022), recently established no differences in protein identification based on pool size at this age range using MS^e instrumentation. For the current study, 20 embryos were chosen to allow for parallel analysis of behavior and gene expression, in addition to proteome characterization.

Briefly, standardized toxicology experimental methods were used for zebrafish (OECD, 2013) and fathead minnows (US EPA, 2002). Solutions of > 98 % pure racemic (±) anatoxin-a fumarate (Abcam, Cambridge UK;CAS 6428506–9) and > 95 % pure caffeine (Sigma-Aldrich, St. Louis MO; CAS 58–08-2) were used in testing. As ANTX-a is an ionizable weak base, solutions were titrated to pH 7.5 for consistency of ionization state (Nichols et al., 2015; Valenti et al., 2009). Water quality parameters (dissolved oxygen, temperature, conductivity, alkalinity, hardness) of treatment levels in reconstituted hard water (American Public Health Association, 1999), which also served as a negative control, were routinely monitored during the experiments. Zebrafish embryos (n = 26 per beaker) were exposed at 4 – 6 hpf, with the majority at 4 – 4.5 hpf, and placed in 100 mL glass beakers with 52 mL of exposure water (n = 4 beakers per treatment level, 2 mL per embryo), and incubated at 28 °C. Fathead minnow larvae (n = 15 per beaker) < 48 h post-hatch at time of the experiment were placed in 500 mL glass beakers containing 300 mL of exposure water as one experimental unit (4 beakers per treatment, 20 mL per larvae) and incubated at 25 °C. For both species, seperate incubators were maintained on backup power with the photoperiod for both species 16-h:8-h light:dark.

Both species were exposed to nominal concentrations of (±) ANTX-a based on environmental exposure distributions, with the highest concentration of 1500 μg/L corresponding to the 97th centile range (Lovin and Brooks, 2020). Nominal treatment levels ranged from 10 to 1500 μg/L for both species initially, with a follow up experiment with zebrafish consisting of an additional 3000 μg/L treatment level. (±) ANTX-a and caffeine (0.412 mg/L for zebrafish and 56.38 mg/L in fathead minnow) were both dissolved in reconstituted hard water (RHW), and exposed for 96 h, with water changes occurring daily for zebrafish and at 48 h for fathead minnow. Caffeine was previously shown to inhibit acetylcholinesterase (Pohanka and Dobes, 2013), but also as acts as a neuro-stimulant in both zebrafish and fathead minnow larval exposures (Steele et al., 2018b). After 96 h exposure, larval zebrafish and fathead minnow were collected from each beaker, washed twice with PBS and snap frozen in liquid nitrogen.

2.3. Sample preparation

Total RNA and protein was simultaneously extracted from 21 to 24 zebrafish and 13–15 fathead minnow larvae per experimental unit/beaker (n = 4) as outlined previously (Lovin et al., 2021) with protein stored at −80 °C. Specifically, total RNA and protein were simultaneously extracted using the AllPrep RNA/Protein kit (Qiagen, Germany), following manufacturer's instructions. Protein was thawed on ice within days of the experiments and quantified using the Pierce BCA assay (ThermoFisher). Furthermore, for rapid evaluation of the quality and reproducibility of the extractions among the biological replicates, each sample was first inspected using SDS-PAGE (100 μg) followed by Coomassie blue staining. The protein was prepared using the filter aided sample preparation (FASP) method as previously described (Wiśniewski, 2016) with some minor modifications. Following sample cleanup, reduction with DTT and alkylation with IAA (0.5 M), protein samples were digested with Trypsin/Lys-C mix (Promega, V5073) at a 1:100 ratio (1 μg trypsin to 100 μg protein) overnight [18 h] at 37 °C. Tryptic peptides were recovered by centrifugation, rinsed with 0.5 M NaCL, centrifuged, acidified with 5 % trifluoroacetic acid (TFA) and collected. The tryptic peptides were subsequently purified using the Stop-and-go-extraction (STAGE) tip procedure as previously described (Rappsilber et al., 2003).

2.4. Data acquisition and processing

All samples were analyzed by LC–MS using a nanoAcquity UPLC™-MS^E system coupled to a Waters Synapt G2 quadrupole-time-of-flight (QTOF) mass spectrometer (Waters, Millford, USA). The LC–MS/MS system was operated via the MassLynx control software suite (Version 4.2, Waters). Samples (2 μL) were applied to an on-line LC pre-column trap by partial loop injection with the aid of the autosampler component of the Acquity M−class system. Peptides were eluted from the pre-column trap (Symmetry C18, 100 Å 5 μm, 180 μm × 20 mm, Waters Corporation) and separated on a nano flow UPLC column (HSS T3 1.8 μm, 75 μm × 250 mm, Waters Corporation) with a 95 min linear gradient of 2–30 % mobile phase B (methanol 0.1 % formic acid) and mobile phase A (water, 0.1 % formic acid) at a flow rate of 450 nL/min for fathead minnow, and a 65 min linear gradient for zebrafish. The eluted peptides were introduced into the mass spectrometer on-line via a nano electrospray source with temperature set at 80 °C and capillary voltage of 2.6 kV. The mass spectrometer was set to efficiently transmit ions with $m/z > 300$ and the TOF to detect ions in the 200–2000 m/z range. Following each sample run, the column was immediately recalibrated using a 65 min sample run with 0.1 % formic acid/methanol solution. The lock mass, [Glu-1]-fibrinogen [100 fmol/μL, 785.8426], was delivered from the auxiliary pump of the LC system at 1 μL/min to the reference sprayer of the NanoLockSpray source every x minute. The acquisition method included ion mobility separation, which preceded peptide parent ion fragmentation in the transfer cell. The instrument was set to allow the collision energy to cycle between low and high values to generate full-scan MS and fragment MS/MS spectra without parent ion isolation, which represents a data-independent MS/MS acquisition mode referred to as MS^E. The low collision energy was set at 6 V and the high collision energy was set to be drift time-specific and ramped during acquisition from 17 V to 60 V. Samples were analyzed in quadruplicate. Yeast enolase peptide digestion standard (Waters) was run at the start, middle and end of sample runs to evaluate HPLC column and mass spectrometry instrumentation.

2.5. Peptide identification and bioinformatics

All data was prepared as previously outlined (Langan and Brooks (2022). Briefly, waters MS^E files were converted to ".mzML" using msconvert software [v 3] (Chambers et al., 2012), first running 'lockmasRefiner' and then scanEvent [1–2] to remove all lockmass spectra. Thereafter, all files were centroided using MSNbase [v 2.15.7] (Gatto et al., 2020), and peak picking, retention time alignment and grouping carried out using the 'xcms' package [v 3.12] (Benton et al., 2010; Smith et al., 2006; Tautenhahn et al., 2008) with treatment and species specific spectra parameters chosen using the 'patRoon' package [v 2.2.0] (Helmus et al., 2021). The raw data files were searched against the zebrafish fish proteome UP000000437 (UNIPROT; 46,849 sequences) or from the fathead minnow proteome (EPA FHM 2.0; 47,578 protein sequences (Martinson et al., 2022) using the de Bruijn decoy generation tool (Moosa et al., 2020) and the target-small decoy search strategy (Kim et al., 2019). For each database, a contaminant protein database cRAP was amended to each file prior to the derivation of decoys. Peptide identification was carried out on the extracted peaks using MSGF+/MSGFPlus [release 2021.01.08] (Kim & Pevzner, 2014) and then peptide spectrum matches identified in the samples were subsequently rescored by Percolator using default settings (Käll et al., 2007; Spivak

et al., 2009; The et al., 2016). Search parameters are outlined in Table S1 with the false discovery rate (FDR) determined using the target-decoy search strategy. Resulting protein identifications were filtered at 5 % Q value (5 % FDR) for annotated proteins and assembled into protein groups following the principles of parsimony (Occam's razor) (Zhang et al., 2007). Quantification was performed based on total ion current (Asara et al., 2008).

Exploratory data analysis consisted of the use of the 'protti' package [v 0.5.0] (Quast et al., 2022) for each species, individually. Briefly, coefficient of variation among replicates per treatment level was examined, followed by examination of the number of protein identifications and coefficient of variation per replicate sample and treatment grouping, proteome coverage per sample and the whole experiment, before finally examining if the data followed a normal distribution. To check how similar or different the treatment levels were, in addition to how comparable the replicates were, a principal component analysis (PCA) was based on the identified proteins. Complying with the FAIR data principles (Wilkinson et al., 2016), raw and processed data files are made publicly available (See SI material). Raw files were deposited to the ProteomeXchange Consortium via the MASSIVE partner repository with dataset identifier https://doi.org/10.25345/C5707X010.

2.5.1. Concentration - response analysis

Filtered data, consisting of percolator identified proteins with an FDR of 5 %, normalized using the 'median normalization method," which scales the samples to have the same median, and log2-transformed abundances were subjected to concentration - response analysis using the "protti" package [v 0.5.0] (Quast et al., 2022) that uses the log-logistic model with four parameters from the "drc" package [v 3.0–1] (Ritz et al., 2015) using modified parameters. Specifically, data consisting of (±) ANTX-a exposed fish in addition to the negative control were used to construct the concentration - response curves based on protein expression, with the positive control samples consisting of caffeine excluded. To be considered significant, Pearson's correlation coefficient (r) was used to assess the strength of the sigmoid trend of concentration - response profiles, and only peptides which fulfilled the Benjamini-Hochberg-corrected p-values (q-values) < 0.05 obtained from an analysis of variance (ANOVA) and Pearson's correlation coefficients $r > 0.7$ were considered (default parameters).

Due to an electrical storm during sample run, batch effects were introduced in the first 6 samples (negative control and 1950 μg/L), and later for the second follow up experiment in zebrafish. Therefore, prior to differential expression, intensity signal was log2 converted and batch effects corrected using the "proBatch" package [v 1.3.0] (Čuklina et al., 2021). Specifically, continuous drift in the signal was corrected using LOESS regression, and discrete corrections carried out using median centering using default settings. Thereafter, imputation of the missing peptides was carried out using a combination of maximum likelihood estimate (MLE) for missing at random data (MAR), and for missing not at random data (MNAR), probabilistic minimum (MinProb) was used, following recommendations for a combinational MAR/MNAR approach by Gardner & Freitas, (2021). Differential expression was carried out using the 'DEP' package [v 1.20.0] (Zhang et al., 2018).

2.5.1.1. Protein functional annotation and enrichment analysis. Data exploration, visualization and dimensional reduction analysis consisting of the differentially expressed proteins (DEP's) identified ($p < 0.05$, 5 % FDR) from both species were performed using the "mixOmics" package in R (Rohart et al., 2017). Variance in the normalized DEP's were first visualized using principal component analysis (PCA) [unsupervised] using Log2 converted ratios prior to imputation of missing peptides as outlined in prior section. Specifically, for the purposes of examination of variance among treatment levels and replicates, "zero" imputation as carried out. Sparse partial least square discriminant analysis (sPLS-DA) was also performed to visualize and quantify treatment separation between the species.

For fathead minnows, functional annotation of protein groups is currently limited, therefore DEPs were functionally annotated using eggNOG-mapper (Huerta-Cepas et al., 2019), with functional analysis and enrichment of gene ontologies carried out using the "clusterProfiler" package, functional annotation (zebrafish only), and for both species, gene over-representation, KEGG pathways and GSEA analysis were carried out using the "clusterProfiler" package in R (Wu et al., 2021; Yu et al., 2012), with organism annotation packages downloaded via "AnnotationHub" package in R (Morgan and Shepherd, 2023). For background gene sets to test for functional enrichment analysis, the background gene list within the current study was defined as all proteins identified at 5 % FDR within the experimental dataset for each species, as per suggestions to improve reproducibility (Wijesooriya et al., 2022). Furthermore, to identify all pathways pertinent to organismal response to (±) ANTX-a exposure, separate analysis of up- and down-regulated proteins was carried out, in line with prior recommendations (Hong et al., 2014).

2.5.1.2. Pathways analysis. To contextualize the identified proteins with human information, DEPs identified in zebrafish (5 % FDR) were mapped to human equivalents using the "orthogene" package in R. Over-representation and pathway-topology was performed using "Reactome" [version 83, 29/03/2023], a curated and peer-reviewed knowledge based of biomolecular pathways (Fabregat et al., 2016). Using the controlled vocabulary provided by the Medical Subject Headings (MeSH) for indexing articles in PuBMed, the resulting human proteins were converted to genes and subjected to MeSH enrichment analysis using the R package "meshes" (v1.26.0) (Yu, 2018). The p-value was adjusted using the default settings of Benjamin-Hochberg [BH] false discovery rate (FDR). As fathead minnow is not currently a supported species, this was not carried out with this species. Finally, as low level exposure to some cyanobacterial toxins have been reported as carcinogenic, in addition to a rich source of cytotoxic compounds (IARC, 2010; Žegura et al., 2011), identified human orthologues were queried against DisGeNET (Piñero et al., 2015), one of the largest and most comprehensive repositories of human gene-disease associations, comprising 21,671 genes and 30,170 diseases/traits (As of September 2023), in addition to associations for mendelian, environmental and rare diseases and disease-related traits.

2.6. Statistical analysis

While all experimental units initially included 26 and 15 embryos/larvae per beaker for zebrafish and fathead minnow, respectively, due to pooling per beaker, "n" refers to the number of replicate beakers per treatment ($n = 4$). Statistical analysis and graphing were carried out in R (Version 4.3.1, "Beagle scouts") (R Development Core Team 2013).

3. Results

Analytical verification of experimental treatment levels were confirmed in our prior study (Lovin et al., 2021) with slight differences reported between the nominal and measured concentrations. In the current study, only measured concentrations were used for all subsequent analysis, specifically during the exploratory analysis stage, dose response analysis and differential expression.

3.1. Protein group identification

3.1.1. Zebrafish

In zebrafish, identified contaminants were removed and identified organism specific protein groups ($n = 5135$) were filtered based on q-value output from Percolator resulting in 3959 proteins identified at a 5 % FDR and 2954 proteins identified at a 1 % FDR. For all subsequent

analysis, only proteins identified at the 5 % FDR were used. Quality control was assessed using the 'protti' package, with coefficient of protein identifications increasing with exposure concentration, ranging from 70 to 103 % (individual protein identifications) in line with variable numbers of embryos being pooled per replicate due to death (see Table S2) and increasing treatment concentrations. Higher inter-sample variability was observed in the second follow up experiment of zebrafish to 3490 μg/L at 99–114 %. Importantly, coefficient of variation between protein identifications per replicate treatment was between 1 and 11 %, with protein identifications ranging from 2262−2774, treatment dependent (Table S2, Fig. S1A). Principle component analysis of the 3959 proteins identified (5 % FDR) clearly separates the two experiments, with 12 % of the variance explained by the treatment concentration, with overlap of ANTX-a and caffeine exposed fish observed for both experiment 1 (412 μg/L) and 2 (48,460 μg/L), with overlap observed with the positive control during both experiments, and controls during experiment 2. Focusing on variance in the ANTX-a exposure fish alone, a supervised clustering ML model (sPLS-DA) was applied and able to discriminate between negative controls and exposure with a significant separation of the controls and the upper treatment concentrations observed by plotting the principal component (PC)-3 against PC-1 explaining 12 % of the variance, with concentrations 10 – 1310 μg/L overlapping. For the upper treatment concentration of 3490 μg/L, the proteins A0A8M1RLC1 (Gamma-tubulin complex component 6), A0A8M3AVY5 (LIM domain only protein 7 isoform X2) and F1RDB1 (Interferon-induced very large GTPase 1-like) were identified in the top 10 features significantly contributing to the separation (all down-regulated), and were interesting also observed to contribute to the clear separation of the positive control of 48,460 μg/L (up-regulation of A0A8M3AVY5 and F1RDB1) from the ANTX-a exposure. For the controls, the protein A0A2R8PWT8 (Signal-induced proliferation-associated 1-like 1) and A0A8M1Q764 (Zinc finger CCHC domain-containing protein 14) were identified as less expressed in the ANTX-a exposed fish compared to the controls. Interestingly, only F1RDB1, a protein linked to post-translational modifications, was identified as being significantly differentially expressed in the 48,460 μg/L caffeine exposure. For both experiments, the number of detected proteins (expressed as a percentage) identified per treatment did not vary, ranging from 65 to 68 % for anatoxin exposed samples and 68–69 % for caffeine exposed samples (Fig. S1B).

3.1.2. Fathead minnow

In fathead minnows, identified protein groups were filtered (n = 8039) based on q-value output from Percolator resulting in 6426 and 5568 protein group identifications, at 5 % and 1 % FDR cut-off. As per the zebrafish, for all subsequent analysis, only proteins identified at the 5 % FDR were used. The number of proteins identified per sample were broadly similar across treatment levels ranging from 2781 − 3184, treatment dependent, with an average coefficient of variation between replicates of 2–27 %. Two high coefficient of variation treatments (18 + 27 %) were identified, with an irregularity observed in one treatment sample (control, replicate 3) attributed to a pump issue during sample run time. However, on average, protein identifications at 5 % FDR were broadly similar across treatment groups (Fig. S1C). Principle component analysis of the 4058 identified proteins (5 % FDR) separated the negative control and positive control (caffeine) from all ANTX-a treatments (Fig. 1A), although this separation was further improved when sPLS-DA was applied focusing on proteins which were uniquely associated with the negative control, positive control and the anatoxin exposure (Fig. 1B). Narrowing down to the top 10 proteins that differentiate among treatment levels in the experiment, ANTX-a was separated from the positive and negative controls best by increased expression of XP_039520082.1 (DnaJ (Hsp40) homolog, subfamily C, member 8) and XP_039522201.1 (uncharacterized protein K02A2.6-like), while for the negative control, the protein XP_039543952.1 (ubiquitin-conjugating enzyme E2 G1-like) was more expressed than in ANTX-a exposed treatments. Interestingly, XP_039520082.1, XP_039522201.1 and XP_039543952.1 were all later identified as differentially expressed. Finally, the total identified proteins per treatment did not vary substantially (Fig. S1B) while percentage of detected proteins identified per treatment also did not vary substantially (Fig. S1D), ranging from 70 to 77 %.

3.2. Concentration - response analysis and differential expression

To identify proteins that were specifically linked to ANTX-a induced proteome changes, the positive control of caffeine was removed from dose-response analysis for both species but included in the identification of differentially expressed proteins.

3.2.1. Zebrafish

Of the identified proteins which were fitted and ranked based on data completeness (replicate completeness and condition completeness = 50 %) and significance cutoff based on an adjusted p-value obtained from ANOVA ($p < 0.05$), 48 proteins representing 33 % of all proteins that met the criterion were identified in zebrafish (Table S4), with the top 5 identified proteins displayed in Table 1. Many of these proteins displayed reductions in identified protein expression with increasing concentration. Each differentially altered protein had an EC_{50} value (ANTX-a concentration necessary to cause half of the maximum possible effect) associated, ranging from 0.15 to 7975 μg/L, for zebrafish exposed embryos.

3.2.1.1. Differential expression. Following batch correction, over 213

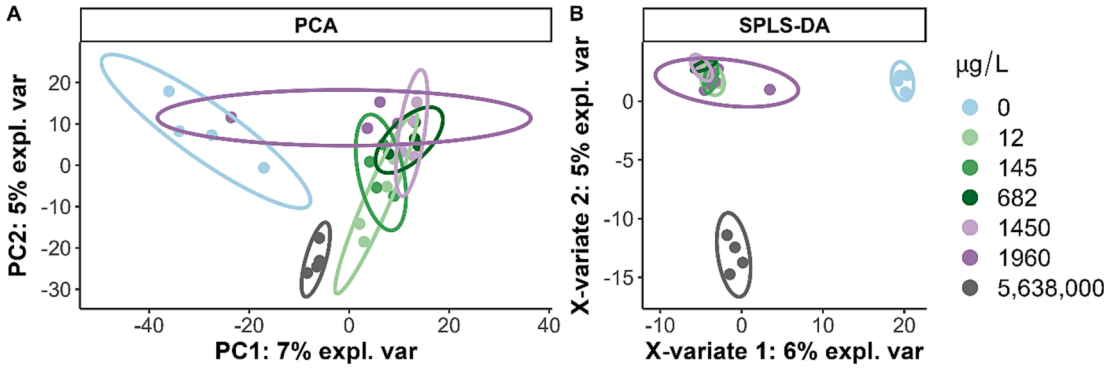

Fig. 1. Principal component analysis (PCA) of proteins identified in fathead minnow (A), with separation of the caffeine positive control. Sparse partial least squares discriminant analysis (with 95 % confidence ellipses) were further applied to the data to focus on discriminate features which may be associated with anatoxin-fumerate exposure response (B) clearly separating negative and positive (caffeine; 5,638,000 ug/L or 56.38 mg/L) controls in addition to grouping anatoxin-fumerate treatment levels.

Table 1
Following the ranking of protein groups based on correlation and ANOVA q-value for zebrafish and fathead minnow, the top 5 are summarized. Excluding *rpl18a* in zebrafish which increases with increasing concentrations (exponential regression model), all other proteins identified decrease with increasing concentrations of anatoxin-fumerate. Note the hill co-efficient (slope), with many slopes > 1, indicating a steep sigmoidal curve. Individual slopes per protein, and all proteins identified as significant can be found in supplemental information (Table S4 & S5). Effective concentration (EC_{50}) is reported for each species, per significant protein group for anatoxin-fumerate concentrations which elicit a response halfway between the baseline and maximum response. Significance is set at $p < 0.05$ and proteins are ranked based on correlation and ANOVA q-value set at < 0.05.

Species	Name	*Abbreviation*	*Hill coefficient*	*EC_{50} (µg/L)*	*Correlation*
Zebrafish	Nucleolar protein 10	*nop10*	8.82	2260.49	0.99
	ISG15 ubiquitin-like modifier (si:rp71-1c23.2)	*isg15*	0.09	36.03	0.94
	Kinesin-like protein KIF22	*kif22*	5.86	6223.64	0.92
	60S ribosomal protein L18a	*rpl18a*	−3.99	4771.25	0.93
	BAH and coiled-coil domain-containing protein 1-like isoform X2	*LOC797750*	4.69	2012.6	0.94
Fathead minnow	uncharacterized si:ch211-107e6.5	*si:ch211-107e6.5*	−3.28	4010.32	0.93
	bloodthirsty-related gene family, member 9	*btr09*	1.6	12.13	0.96
	PR domain containing 8b	*prdm8b*	2.46	332.04	0.94
	uncharacterized LOC120478948	*LOC120478948*	5.95	434.88	0.95
	nuclear receptor subfamily 2, group F, member 6a	*nr2f6a*	0.52	0.37	0.89

proteins were identified as differentially expressed for the full experiment, with 145 DEPs identified specific to the ANTX-a experiment ($p < 0.05$, 5 % FDR), with a general trend towards downregulation with an approximate breakdown of 72 % downregulated (ANTX-a) and a general trend towards increasing numbers of down-regulated proteins with increasing treatment level. A breakdown of DEPs per treatment is provided in Table S6, with only three concentrations of ANTX-a resulting in significant DEPs (671, 1950 and 3490 g/L), although both caffeine exposures resulted in DEPs. Unique DEPs increased with increasing concentration, with only one protein identified in common with all ANTX-a concentrations (Q7ZSY3; Brahma protein-like protein 1 [*smaraca4*]) (Fig 2A). For caffeine exposed fish, only one protein was found to overlap between the two exposure concentrations, A0A8M2B3N0 (Ubiquitin carboxyl-terminal hydrolase) (Fig S2A). When paired with caffeine, 3 proteins (A0A8M9PL68, Q08CI6 and Q5PRA8) were found to overlap between the positive control and anatoxin-fumarate exposure in experiment 1 (Fig S2B), with 17 proteins found to overlap in experiment 2 (Fig S2C), although no proteins were identified to overlap for the full experiment (Fig S2D). For all subsequent analysis, caffeine was removed.

3.2.1.2. Pathways analysis. Level 2 gene ontology grouping of DEPs resulted in 2 cellular components (CC), 17 biological processes (BP) and 11 molecular functions (MF) (Table S7), although no enrichment was identified. KEGG pathways analysis revealed that the "Fanconi anemia pathway" was the only one significantly ($p < 0.05$, 1 % FDR) enriched in the up-regulated proteins identified in the 3490 µg/L alone, with two proteins associated with this enrichment (DNA excision repair protein ERCC-1 [Q6NY87] and Crossover junction endonuclease MUS81 [Q7SXA9]). However, no gene sets or KEGG modules were found to be enriched ($p > 0.05$).

Reading across from zebrafish DEPs identified following exposure to ANTX-a to human based responses, Reactome pathway over-representation analysis mapped the DEPs identified in the zebrafish experiment to 15 Reactome pathways across two different concentrations viz, 671 and 3490 µg/L exposed fish ($p < 0.05$, 5 % FDR), with 14 of these terms associated with the highest treatment level alone (Table S8). All pathways were enriched for up-regulated terms, except 671 µg/L, which linked down-regulated proteins to the "Neddylation" pathway (Fig 2B), a post-translational modification that modulates many essential cellular functions and biological processes, including tumorigenesis. In the up-regulated proteins, gene pathways enriched for DNA repair and Fanconi Anemia pathway are particularly increased in the higher concentration of 3490 µg/L suggesting the presence of DNA damage, which agreed with the KEGG pathway analysis.

To assist with gene-function interpretation, disease ontology (DO) enrichment was carried using DEP converted human orthologues (n = 44 proteins) against a background of human orthologues of the total proteins identified in the zebrafish experiment (n = 931 human proteins). Over 45 diseases were identified with 1 % FDR (Table S9), with 5 diseases having gene counts over 2 (Table 2), and diseases associated with only up-regulated DEPs visualized in Fig 2C. Notably, over 58 % of the diseases were linked to modulation of SWI/SNF Related, Matrix Associated, Actin Dependent Regulator Of Chromatin, Subfamily A, Member 4 [SMARCA4], with a further 7 % associated with modulation of excision repair cross-complementation group 1 [ERCC1]. In parallel, gene set enrichment using the DisGeNET collection also identified three disease categories via Azoospermia (C0004509), primary peritoneal carcinoma (C1514428) and recurrent ovarian cancer (C0278689) as significantly enriched ($p < 0.05$, 5 % FDR), with two genes observed in common with all identified diseases (ERCC excision repair 1, endonuclease non-catalytic subunit (*ERCC1*) [2067] and poly(ADP-ribose) polymerase 2 (*PARP2*)[10038]), and one unique gene only identified in Azoospermia (OCRL inositol polyphosphate-5-phosphatase (OCRL) [4952]). Finally, to assist with the biological interpretation of the experiment relative to humans, MeSH over-representation analysis identified ($p < 0.05$, FDR = 5 %) two anatomical parts linked to ANTX-a exposure (vestibular nerve [D014725] and nephrons [D009399]), two diseases (Vestibular diseases [D015837] and neurotoxicity syndromes [D020258]), two organisms (*Chlamydomonas reinhardtii* [D016825], *Aspergillus nidulans* [D001233]), one process (CRISPR-Cas Systems [D064113]) and genes associated with the "HapMap Project" [D060148].

3.2.2. Fathead minnow

Of the identified proteins that were fitted and ranked based on data completeness (replicate completeness and condition completeness = 50 %) and significance cutoff based on an adjusted p-value obtained from ANOVA ($p < 0.01$), 29 protein groups were identified in fathead minnow (Table S4), representing approximately 23 % of the proteins that met the test criteria. Each differentially altered protein had an EC_{50} value (ANTX-a concentration necessary to cause half of the maximum possible effect) associated, ranging from 0.36 to 4010 µg/L, which was less than half of what was reported for zebrafish, suggesting increased sensitivity of this species to ANTX-a.

3.2.2.1. Differential expression. Following ANTX-a exposure to fathead minnow, over 427 differentially expressed proteins were identified, with 300 of these proteins associated with ANTX-a exposure ($P < 0.05$, 5 % FDR). Unlike zebrafish, DEPs were associated with all exposure concentrations, with 86 % of the total proteins identified downregulated, and when limited to DEPs identified in ANTX-a exposed fish increased to 90 %. A full breakdown of differentially expressed proteins per treatment is provided in Table S6. Principle component analysis of the DEPs

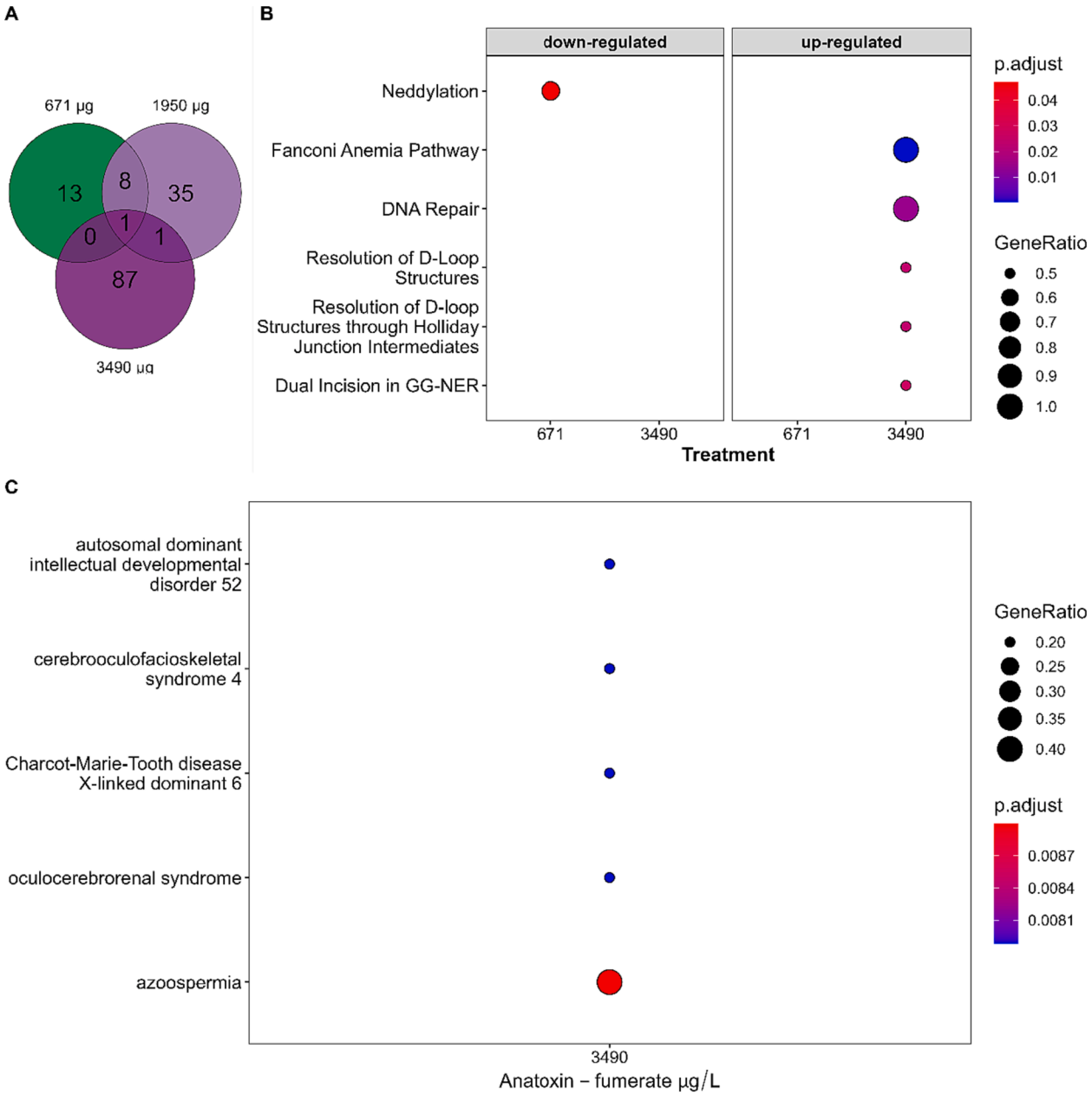

Fig. 2. Venn diagram of overlapping DEPs following exposure to anatoxin-fumerate in zebrafish in the three treatments identified as significantly different from control (A). Following conversion of DEPs identified in zebrafish to human orthologues, Reactome pathways over-representation identified 15 enriched terms (B) the majority (n = 14 pathways) linked to up-regulated proteins in zebrafish. Finally, Disease ontology was used to translate the DEPs identified in zebrafish to human clinical relevance, with enriched disease terms found in both the up and down-regulated groups (n = 45), with the diseases associated with the up-regulated DEPs outlined (C). In supplemental information, the full list of terms enriched are outlined for enriched Reactome pathways (Table S8) and diseases (Table S9). Significance was set at $p < 0.05$ with 5 % FDR applied.

Table 2
Following conversion of DEPs identified in zebrafish to human orthologues, Disease Ontology Semantic and Enrichment analysis (DOSE) analysis was carried out on human orthologs of zebrafish DEPs identifying 45 diseases associated with DEPs identified in zebrafish following anatoxin-fumerate exposure, with diseases annotated with more then one gene outlined below($p < 0.05$, 1 % FDR). Notably, approximately 58 % of the diseases were linked to modulation of SMARCA4, with a further 7 % associated with modulation of ERCC1.

Exposure	*group*	*ID*	*Description*	*GeneRatio*	*BgRatio*	*geneID*
1950	Down-regulated	DOID:630	genetic disease	1/3	208/10312	CHD7/SMARCA4
1950	Down-regulated	DOID:0060041	autism spectrum disorder	1/3	321/10312	CHD7/PHF8
1950	Down-regulated	DOID:0060040	pervasive developmental disorder	1/3	334/10312	CHD7/PHF8
1950	Down-regulated	DOID:1059	intellectual disability	1/3	389/10312	PHF8/SMARCA4
3490	Up-regulated	DOID:14227	azoospermia	1/3	87/10312	PARP2/ERCC1

(5 % FDR) reveals that ~ 49 % of the variance was explained by the top 10 DEPs. Unique DEPs were consistent across the exposure concentrations at approximately 22–35 proteins, with 60 proteins shared among all the treatment levels, and the highest number of unique proteins identified at the 145 µg/L treatment (Fig 3A), in contrast to the zebrafish results. For all samples from the experiment, including the caffeine treatment, 46 protein groups were shared. Focusing on the shared proteins identified among the ANTX-a exposed fish, this list was additionally used for experimental pathways analysis via GO analysis and KEGG, since little is known about how other aquatic fish species react to ANTX-a exposure. For all subsequent analysis, caffeine was again removed.

Following eggNOG functional category annotation of fathead minnow differentially expressed proteins, level 2 gene ontology grouping of DEPs resulted in the same 2 cellular components (CC) as zebrafish (viz. cellular anatomical entity [GO:0110165] and protein-containing complex [GO:0032991]), although increased GO groupings were identified in fathead minnows at 25 biological processes (BP) and 25 molecular functions (MF) (Table S7). Notably, fathead minnows shared 11 MF GO terms with zebrafish, with 14 unique in addition to 17 shared BP GO terms, and 6 unique to this species. Gene ontology (GO) enrichment analysis using total proteins converted to gene ontology identifications via EGGNOG as our background (n = 3281) were carried out on both the up and down regulated proteins. In addition, due to limited GO information, the zebrafish annotation package was used to contextualize the results, since following fathead minnow annotation via eggNOG, over 80 % of GO terms were associated with zebrafish. Stringent criterion of $p < 0.01$ and a p-value cutoff on enrichment tests of 0.01 were applied, with significant enrichment found across all categories in only the down-regulated DEPs (Fig S3), while upregulated DEPs were only enriched in BP and MF, with only GTPase activator activity identified in the molecular function process (Fig 3B). KEGG pathways analysis revealed that the "ATP dependent chromatin remodeling" and "ABC transporters" were significantly enriched in only two treatment levels (Fig 3C) ($p < 0.05$), with variable proteins

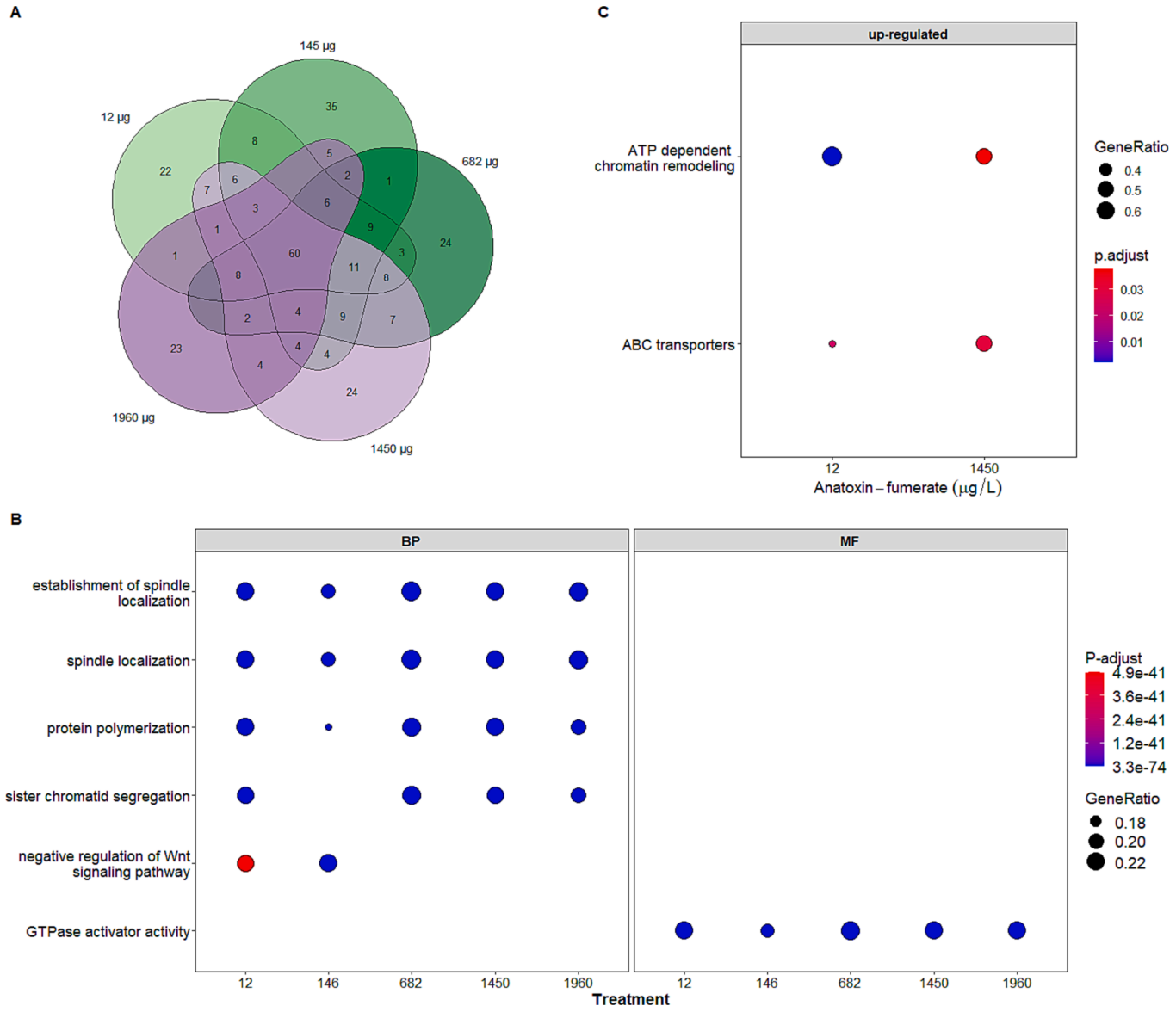

Fig. 3. Venn diagram of overlapping DEPs following exposure to anatoxin-fumerate in fathead minnow in all treatments identified as significantly different from control (A). A dot plot shows the up-regulated GO terms of DEPs identified in fathead minnow (B) with the down-regulated cohort displayed in SI (Fig S3). Finally, enriched KEGG pathways associated with 2 treatment levels for fathead minnow are displayed (C). The color of the dots represents the p-adjusted values (BH), while Gene Ratio represents the fraction of differentially expressed genes identified in the gene set. Significance was set at $p < 0.01$ with 1 % FDR applied for gene ontology enrichment.

identified per treatment level. Three proteins were associated with this enrichment: SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 1 (*smarca1*), chromodomain helicase DNA binding protein 4b (*chd4b*) and ATP-binding cassette sub-family A member 5-like (LOC120474739). However, as with zebrafish, no gene sets, or KEGG modules were found to be enriched ($p > 0.05$).

4. Discussion

Increasing in duration, magnitude, and frequency, algal blooms that contaminate freshwater and coastal resources with cyanotoxins constitute a globally concerning occurrence. ANTX-a is reportedly one of the most frequently detected cyanotoxins in freshwater (Quiblier et al., 2013), and despite its reported adverse effects, this neurotoxin is understudied, compared to other cyanotoxins such as microcystin-LR (MC-LR). Studies conducted under well characterized laboratory conditions can provide enhanced understanding of the direct effects of this toxin and are increasing, although at a slow rate. Recently, Plata-Calzado et al., (2022) summarized the toxic effects of ANTX-a under laboratory conditions of both terrestrial and aquatic organisms reporting limited publications of both *in vitro* and *in vivo* studies over the past 4 decades. In aquatic toxicology, studies focused on understanding ANTX-a (synthetic, purified from culture and from crude uncharacterized cell extracts) are sparse, with 20 aquatic toxicity articles and 5 bioaccumulation studies meeting filtering criteria in a recent review (Lovin and Brooks, 2020). Notably, aquatic toxicology studies are typically carried out using crude uncharacterized cultured extracts potentially containing many other potentially stimulatory compounds (n = 6 studies), ANTX-a of unreported origin (n = 2 studies), or the synthesized racemic (±) anatoxin-a (n = 4 studies), with large variations in toxicological response reported. For example, Oberemm et al. (1999) reported no effects on the development of zebrafish following immersion in 400 μg/L of pure and crude ANTX-a, while Rogers et al., (2005) observed a concentration dependent transient narcosis, edema and loss of balance in addition to 100 % mortality in toad embryos exposure to 0.03–30 mg/L of ANTX-a. More recently, Lovin et al. (2021) identified significant differences in both behavioral endpoints in addition to gene expression responses for two fish species exposed to (±) ANTX-a, suggesting species specific responses may be driving observed differences in the literature. Although not for ANTX-a, species specific differences have also been observed in tissue accumulation of MCs in closely related salmonid species (Shahmohamadloo et al., 2022). Research outlined in the current study extends from previous work, building knowledge of how exposure to this cyanotoxin may modulate the proteome of two fish model commonly used by both the biomedical and ecotoxicological communities.

4.1. (±) Anatoxin-a induces variable protein-based concentration - response

Whereas proteomic studies characterizing fathead minnows are limited (Lavelle et al., 2018; Martyniuk et al., 2012, 2009, 2009; Martyniuk and Alvarez, 2013), larval studies are even more sparse (Moreton et al., 2020; Langan and Brooks, 2022). Following the application of a four-parameter log-logistic concentration - response curve for both species, the number of protein groups meeting the criterion did not differ between species, although the top protein identified did. For zebrafish, nucleolar protein 10 (nop10/nol10) was highly correlated with treatment level (0.99), with a resulting EC_{50} calculated as 2260 μg/L. Following exposure to (±) ANTX-a, zebrafish expression of nol10 decreased substantially after 1450 μg/L, with no detection at higher treatment levels. Phylogenetic-based propagation of functional annotations within the gene ontology consortium (Gaudet et al., 2011) has proposed this as being located and active in the nucleus of zebrafish, and part of small-subunit processome, with increased expression in the 5 dpf embryo, adult ovary and adult female head. Functionally a protein coding type, it is reportedly orthologous to human NOL10, although this protein is identified in over 27 tissues in humans. In humans, it is required for ribosome biogenesis and telomere maintenance, plays a key role in carcinogenesis, with high expression levels in combination with other factors associated with breast cancer progression (Elsharawy et al., 2021) and is required for optimal enzymatic activity (Li et al., 2011). Alternatively, Polyglutamine binding protein 5 (PQBP5) is an essential protein necessary to main the structure of the nucleolus (Jin et al., 2023), with NOL10 depletion reported to promote oxidative stress and disruption of biogenesis in human *in vitro* models (Ibáñez-Cabellos et al., 2020). Furthermore, nucleolar dysfunction has been implicated as a pathological domain for polyQ diseases, which are inherited neurodegenerative diseases, which typically begin in adulthood and progress over 10 to 30 years resulting in death (Paulson et al., 2000). Previously, exposure to (+) ANTX-a has been associated with neurotoxicological effects in fish (Carneiro et al., 2015; Colas et al., 2020; Osswald et al., 2007a), with spontaneous muscle spasms, seizures and neural injuries including neurodegeneration observed in mammals (Banerjee et al., 2014; Puschner et al., 2010). The observation of decreased NOL10 expression at higher treatment levels is supported by the existing pure (+) anatoxin literature, with NOL10 also associated with pathological deformities (Jin et al., 2023). As previously mentioned, variation in the type of ANTX-a used in toxicological studies exists, with (+) ANTX-a reportedly 150 fold more potent than the synthetic enantiomer (-) anatoxin-a (Osswald et al., 2007b), and twice as potent in activating nAChRs compared to the racemic mixture (Spivak et al., 1983). While the current study used the racemic mixture for initial study investigations, the results would suggest that increases in deformities could be observed with exposure to the natural (+) ANTX-a, with long term exposure or even short-term exposure potentially leading to neurodegeneration on an unknown scale. In support of this, prior low exposure levels of MC-LR significantly promoted hepatic inflammation, resulting in nonalcoholic steatohepatitis disease (NASH) (He et al., 2017), with later studies suggesting that co-exposure of MC-LR may increase the risk of non-alcoholic fatty liver disease (NAFLD) progression to NASH (Sarkar et al., 2019) in mice. While there is scant evidence of ANTX-a effects from cyanobacterial exposure and how it affects disease development or progression, the results observed suggest the need for future investigations conducted using pure (+) ANTX-a, which we are currently investigating.

Fathead minnows share comparable transcript and protein abundances with zebrafish, in addition to 93 % homology of transcripts to top-matches genes in the zebrafish genome (Martinson et al., 2022), although prior toxicological studies have demonstrated that both species can behave differently in terms of sensitivity under similar experimental conditions using different laboratory methods (Corrales et al., 2017; Steele et al., 2018a, 2018b). Of the identified proteins with concentration - response relationships that met the study criteria, the Kinesin protein superfamily was identified in both species, although with differing concentration - response relationships but comparable correlations at 0.9 – 0.92. Kinesins are a protein superfamily that belongs to the motor protein group, responsible for the transport of various membrane organelles, protein complexes, and mRNA, in addition to the regulation of intracellular and molecular signal pathways, with 45 genes that encode these proteins identified in mouse and human genomes to date (Hirokawa et al., 2009). Kinesin-like DNA-binding protein (*KIF22*) is a plus-end-directed microtubule-based motor and binds to both microtubules and chromosomes, which participate in the entire process of mitosis (Yu et al., 2014). During human development, *KIF22* proteins are associated with proliferating cells, with levels increasing during the cell cycle and peaking at mitosis. In zebrafish, a 50 % decrease in expression leads to abnormal brain and body shapes (Blaker-Lee et al., 2012), with recent studies highlighting that perturbation of KIF22 in human embryos may affect cell-cycle kinetics, and perturb neurogenesis and neuronal output (Morson et al., 2021). Although developmental

abnormalities were minimal in zebrafish in this study (Lovin et al., 2021), KIF22 was identified to decrease approximately 25 % in expression with increasing concentrations (EC_{50} = 6223 μg/L). Typically, decreased or abnormal locomotor behaviors in response to ANTX-a is observed in both terrestrial (Banerjee et al., 2014; MacPhail et al., 2007; Stolerman et al., 1992) and aquatic organisms (Colas et al., 2020; Osswald et al., 2011; Sierosławska, 2013), with chaotic locomotor behaviors observed in adult zebrafish after a short 5 min exposure (Carneiro et al., 2015). In the behavioral component of our previously reported study (Lovin et al., 2021), stimulatory movement was observed although not significantly. It can be postulated that the lack of comparable behavior in zebrafish embryos may not just be linked to age specific differences but may also be as a function of decreased KIFF22 expression, which has been associated with abnormal brain activity/development with a high exposure required to induce a response. Further work will be required to tease apart its function in organismal response to this neurotoxic cyanotoxin.

Contrastingly, fathead minnow demonstrated increased expression the kinesin family member 3B (kif3b; EC50 = 3835 μg/L) with increasing treatment level. KIF3 is one of the most common expression types, essential in biological processes where it not only regulates cell migration but also regulates the cell cycle process, thus promoting cell proliferation and survival. Critically, KIF3B is highly expressed in the retina in zebrafish, is essential for synaptic transmission in photoreceptor cells through axonal transport through chromosome movement during mitosis and meiosis (Feng et al., 2017) but it also has a critical role as regulator of dendritic development (Joseph et al., 2020), and neuronal differentiation. Further, it is important to acknowledge its close relationship to the occurrence and development of many diseases, as recently reviewed (Zhou et al., 2019). In the first phase of the current research effort, Lovin et al., (2021) observed significantly lowered locomotor behavior, specifically at bursting speeds (>20 mm/s), in fathead minnow across higher ANTX-a concentrations, with proteomics analysis suggesting that kinesins, and specifically KIF3B may have contributed to this behavioral observation. Previous studies have shown following an acute spinal cord injury in adult rats, the expression of KIF3B increased significantly and was closely related to the activation and proliferation of microglia and astrocytes (Yu et al., 2013; Zhou et al., 2017), with recent reviews highlighting the contribution of microglial activation to neuronal damage and neurodegenerative diseases via the release of reactive oxygen species (ROS) (Block et al., 2007). However, while it is unknown if this has such an established link in fish as in humans, the result of the current study in addition to our previous behavioral assays (Lovin et al., 2021) suggests that exposure to ANTX-a resulted in a neurotoxic response and potentially genotoxicity (via ROS) in fathead minnow, which is also in agreement with the literature across numerous aquatic organisms (Bownik and Pawlik-Skowrońska, 2019; Carneiro et al., 2015). However, such observations were not made in zebrafish, at least via these molecular mechanisms. Differences in organismal response between the two fish species, in combination with contrasting kinesin concentration - response expression profile would suggest differences in sensitivity and/or response between the two model organisms. While this has previously been observed between the two species using both gene expression and behavioral assays (Lovin et al., 2021), differences in sensitivity have also been observed in other animal models (goldfish, duck, calf, pheasant, rat and mouse) with the goldfish identified as a sensitive species (Carmichael et al., 1975). It is important to note many proteins were identified in the concentration - response analysis, and that exploration of these other proteins provides an avenue to explore modes and mechanisms of action in both species for the first time.

Defining dose–response relationships with proteomics data affords the possibility to examine proteins that are potentially being modulated together, allowing us to more fully flesh out the pathways impacted by cyanotoxin exposure. However, while several protein groups were identified with large ranges of EC_{50} values, potentially representing a preferential molecular target for this cyanotoxin, based on a recent critical review (Henke et al., 2024) this appears to be the first aquatic toxicology study to fit a dose response relationship to proteomics data from alternative vertebrate models like the fish examined here. Toxicological studies of cyanotoxins, and specifically ANTX-a, are limited, and the application of omics approaches looking at 1000 s of molecular changes even more so. The sublethal toxicity of ANTX-a is poorly understood across the animal kingdom, with this approach potentially leading to a further understanding of the sub-lethal mechanism of action of this poorly understood cyanotoxin. Representing the first application of this approach in aquatic toxicology with an ANTX-a surrogate, future investigations will involve comparing proteins with dose responses in the pure (+) anatoxin-a in order to identify whether these proteins represent a preferential molecular target.

4.2. *Differential protein expression following exposure*

The molecular mechanisms by which both the racemic (±) and natural form (+) of ANTX-a induces toxicological responses during fish development remain largely unknown. Currently, proteome characterization of ANTX-a is limited to one acute exposure study (5 min) via intraperitoneal injection of adult zebrafish using the racemic form, identifying gendered responses (Carneiro et al., 2015) and limited differential expression. The present study is the first to characterize both the proteome zebrafish larval response to a (±) ANTX-a, but also fathead minnows following 96 h of exposure, representing both environmental and potentially human responses to environmentally relevant concentrations of the natural form of ANTX-a (Lovin and Brooks, 2020). Differentially expressed proteins were identified in both species (~145 – 300) at 5 % FDR, with downregulation of proteins observed more frequently, which was in agreement with the available literature (Carneiro et al., 2015). In both species, DEPs were grouped to similar biological processes (viz., cellular, metabolic and biological regulation), but also molecular functions (binding, catalytic activity) with the third top term ATP-dependent activity in zebrafish which is in agreement with the literature (Carneiro et al., 2015), although differences in enrichment was observed between the two species.

ATP dependent chromatin remodeling and ABC transporters were significantly enriched in fathead minnow following exposure to (±) ANTX-a. ATP dependent chromatin remodeling play important, although poorly characterized, roles in facilitating the effectiveness of the DNA-damage response (Lans et al., 2012). As noted above, there is limited evidence examining genotoxic impacts of ANTX-a to organisms, following its designation as a neurotoxin, although genotoxic effects have been observed previously *in vitro* and in bacteria exposed to (±) ANTX-a (Plata-Calzado et al., 2023; Sierosławska and Rymuszka, 2010), but not in common carp leukocytes (Sierosławska and Rymuszka, 2013). Increased REACTOME pathways associated with DNA repair molecular mechanisms in zebrafish, but also in KEGG pathways identified as enriched were identified in the current study. SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily A, member 1 (SMARCA1) protein is an important subunit of the SWI/SNF complex involved in chromatin remodeling and transcriptional activation (Eberharter and Becker, 2004; Goodwin and Picketts, 2018), with the mammalian SMARCA1 implicated in neurodevelopmental disorders (Goodwin and Picketts, 2018) but also in human carcinomas (Dai et al., 2022). In aquatic organisms, SMARCA1 is required in early in haematopoietic (formation of blood cellular components) development in zebrafish (Huang et al., 2013), although it is unknown if it serves the same function in fathead minnows. According to the Comparative Toxicogenomics Database (CTD) (Davis et al., 2013, 2009), chemical-gene interactions with the gene SMARCA1 in fathead minnows return a number of interacting chemicals, with bisphenol A, a potent well-known endocrine disrupter, the chemical with the highest interactions. Similar to our current observation of up-regulation and enriched of SMARCA1 protein in fathead minnows, SMARCA1 mRNA is

increased following exposure to bisphenol-A in both human *in vitro* models of breast cancer and mice cardiovascular function (Belcher et al., 2015; Kim et al., 2019a).

The second enriched protein was chromodomain helicase DNA binding protein 4b (*chd4b),* a protein, which is expressed in the central nervous system of zebrafish and orthologous to human CHD4. In humans, SMARCA1 and CDH4 are part of chromatin remodeling complexes linked to DNA double-strand breaks (Rother and van Attikum, 2017). Based on the combination of up-regulation of these proteins in fathead minnows, it can be postulated that (±) ANTX-a could act as an endocrine disrupting chemical through the dysregulation of DNA repair mechanisms, with a possible similar mode of actions in zebrafish and humans with the identification of genes associated with azoospermia identified following exposure to (±) ANTX-a. As previously introduced, molecular approaches to characterize organismal response to this cyanotoxin, and indeed, other cyanotoxins, is limit, but does suggest gender specific responses following exposure (Carneiro et al., 2015). Although not enriched, differentially expressed proteins (down-regulation) were also identified in both species linked to sperm structure and function (spef2, LOC120481685) at the higher exposure concentration. In a recent review, the impact of the cyanotoxin MC-LR on reproductive toxicology was summarized, with MC-LR and other MCs reported to possibly act as an endocrine disruptor (Wang et al., 2020; Zhang et al., 2021), with Guo et al., (2023) reporting a similar down-regulation of SPEF2 linked to the induction of functional and structural damage in human sperm following exposure to MC-LR. Endocrine disruption has recently been reviewed for MCs and another cyanobacterial toxin cylindrospermopsin (Casas-Rodriguez et al., 2022), although information on ANTX-a is limited. ANTX-a was categorized as an E2 agonist via a yeast estrogen screen assay, and proposed as a potential endocrine disrupting chemical due to their modulation of E2 activity (Liu et al., 2018). Little is known about the affinity of ANTX-a for estrogen receptors, although studies have linked sex hormones and the regulation of nAChR expression in mammals (e.g. Gibbs, 2010; Moen & Lee, 2021), thus adding further support to this suggestion, especially since anatoxin-a is a potent inhibitor of the enzyme acetylcholinesterase (AChE) by binding to neuronal receptors of acetylcholine (nAChR). However, it important to note that up-regulation of these pathways was observed in fathead minnows while analogous protein families in zebrafish were down-regulated, suggesting variation in response and potentially sensitivity of these two common alternative vertebrate models to this cyanotoxin.

Distinctly different responses to (±) ANTX-a were also previously observed with behavioral assays and gene expression (Lovin et al., 2021). The variation in response between the two species may be due to variation in age at time of exposure. As standardized experimental designs from regulatory or international organizations were used in these studies to maximize comparability to other work, the development age of the test species differed during the initiation of the toxicity experiment. CYP-mediated metabolism changes during fish development, which may act as a contributing factor to the observed differences between the two species. For example, older fish (fathead minnows) may have increased biotransformation, resulting in modification of molecular changes. Contrastingly, the differential responses may also be due to differing sensitivities of the two fish species to this cyanotoxin, with prior studies demonstrated marked differences in acute toxicity and gene expression of 8 compounds (Corrales et al., 2017). Given the identification of potential endocrine disruption following exposure to this model laboratory organisms during the larval stage of development, it is evident that molecular characterization of adult responses to this cyanotoxin must be carried out to understand potentials impact of this cyanotoxin on reproductive biology.

4.3. Sparse neuronal nicotinic-acetylcholine receptors (nAChRs) activity

Though sublethal toxicity of ANTX-a is poorly understood across the animal kingdom, it has been reported to bind irreversibly to nAChRs (Molloy et al., 1995; Swanson et al., 1985), with highly variable responses reported in the aquatic literature. Cholinergic nicotinic receptors are common across vertebrates including fish (Zirger et al., 2003), and while cholinergic receptor, nicotinic, alpha 6 (*chrna6*) was down-regulated in fathead minnows in the current study, it was only observed in caffeine and not following cyanotoxin exposure. One possible explanation for this may lie in the duration of exposure and time of sampling. To establish the binding of ANTX-a to the nicotinic acetylcholine receptors, experiments were typically carried out for 5–20 min (Molloy et al., 1995; Swanson et al., 1985), and not hours as in the current studies (~100–102 h, sampled at midday). Interestingly, while the only other proteomic analysis of ANTX-a also exposed for 5 min, no cholinergic receptor activity was identified, although it was identified that adult zebrafish appear to have an increased tolerance compared to mice (Carneiro et al., 2015). Due to the short nature of the exposure in this previous work, it was suggested that post-translational modifications (PTMs) were responsible for the observed changes in protein expression (Carneiro et al., 2015), which is in general agreement with the current study, although other factors should also be considered.

PTM neddylation, most closely related to ubiquitination, was identified as downregulated in zebrafish with neddylation having a diverse biological functions, including functions in the immune system and metabolism as recently summarized (Zou and Zhang, 2021). Critically, PTMs regulate all levels of the nAChR cycle (Chrestia et al., 2023), which is also suggested as being under circadian fluctuation although evidence of diurnal changes are variable among the study organisms. As noted above, the molecular mechanisms of cyanotoxins are poorly understood in general, and even more so at the epigenetics level, although epigenetic mechanisms of response in organisms following exposure to HABs is increasing (Gonzalez-Romero et al., 2017), in addition to epigenetic information associated with algal cultures (Zhu et al., 2022). Together, it is possible that the time of exposure and sampling may also contribute to the variability observed in the literature, in addition to PTMs, which may have also contributed to lack of significant changes at the gene expression level in our recent studies (Lovin et al., 2021).

5. Conclusions

Our proteomic analysis has provided new perspectives on (±) ANTX-a toxicity in the model aquatic organisms examined here, which included a common alternative vertebrate model for biomedical research and a common fish model in ecotoxicology. However, additional research is required to provide a more complete understanding of what pathways are impacted by, and potential adverse outcomes elicited by exposure to ANTX-a. Whereas studies have specifically identified ANTX-a as a neurotoxin, our proteomic characterization of responses to the surrogate (±) ANTX-a has revealed other potential mechanisms by which this toxin could elicit toxicological response (e.g., via endocrine-disruption and PTMs), with the information highlighting the need for the rapid expansion of omics-based characterization in future studies among species. Expansion of toxicological studies using omics-based molecular assays including proteomics, transcriptomics, metabolomic characterization of cyanotoxins across multiple organisms are essential to expanding our understanding of the sublethal effects of ANTX-a exposure, in addition to multiple other cyanotoxins. Using this information, adverse outcome pathways can be pragmatically built to support understanding of the toxicological impacts to humans, fish, and other organisms. Further, future research is needed to expand our understanding of toxicological impact of cyanotoxins, including an often overlooked but essential component is differences in toxicokinetic-toxicodynamic among species (Carter et al., 2024; Brooks et al., 2024), in addition to considering other factors such as age, sex and environmental variables such as light, duration and frequency of exposure. While examples do exist, they are almost exclusively in the study of microcystins (Zhang et al., 2013), which has received relatively more

toxicological attention with aquatic organisms than other cyanotoxins (Mehinto et al., 2021), which can cause health impacts to numerous organisms. Further efforts are necessary to advance a mechanistic understanding of the comparative toxicology of the anatoxins and other cyanotoxins among species.

CRediT authorship contribution statement

Laura M. Langan: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Conceptualization. **Lea M. Lovin:** Writing – review & editing, Investigation, Conceptualization. **Raegyn B. Taylor:** Writing – review & editing, Investigation, Formal analysis. **Kendall R. Scarlett:** Writing – review & editing, Investigation. **C. Kevin Chambliss:** Writing – review & editing, Resources. **Saurabh Chatterjee:** Writing – review & editing, Funding acquisition. **J. Thad Scott:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Bryan W. Brooks:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data generated during this article has been published to a repository with link in the manuscript text.

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Appendix A. Supplementary material

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