

**Molecular networks related to the immune system and mitochondria are targets for the pesticide dieldrin in the zebrafish (*Danio rerio*) central nervous system**

Andrew M. Cowie<sup>a</sup>, Kathleena I. Sarty<sup>a</sup>, Angella Mercer<sup>a</sup>, Jin Koh<sup>b</sup>, Karen A. Kidd<sup>a</sup>, Christopher J. Martyniuk<sup>a,c,\*</sup>

<sup>a</sup> Canadian Rivers Institute and Department of Biology, University of New Brunswick, Saint John, New Brunswick, E2L 4L5, Canada

<sup>b</sup> Interdisciplinary Center for Biotechnology Research, University of Florida, Gainesville, Florida 32611

<sup>c</sup> Current address: Department of Physiological Sciences and Center for Environmental and Human Toxicology, University of Florida Genetics Institute, Interdisciplinary Program in Biomedical Sciences Neuroscience, College of Veterinary Medicine, University of Florida, Gainesville, FL, 32611 USA

\*Corresponding author: CJ Martyniuk

Email: cmartyn@ufl.edu

**Abstract** The objectives of this study were to determine the behavioral and molecular responses in the adult zebrafish (*Danio rerio*) central nervous system (CNS) following a dietary exposure to the pesticide dieldrin. Zebrafish were fed pellets spiked with 0.03, 0.15, or 1.8 µg/g dieldrin for 21 days. Behavioral analysis revealed no difference in exploratory behaviors or those related to anxiety. Transcriptional networks for T-cell aggregation and selection were decreased in expression suggesting an immunosuppressive effect of dieldrin, consistent with other studies investigating organochlorine pesticides. Processes related to oxidative phosphorylation were also differentially affected by dieldrin. Quantitative proteomics (iTRAQ) using a hybrid quadrupole-Orbitrap identified 226 proteins that were different in abundance in one or more doses. These included ATP synthase subunits (mitochondrial) and hypoxia up-regulated protein 1 which were decreased and NADH dehydrogenases (mitochondrial) and signal recognition particle 9 which were up-regulated. Thus, proteins affected were functionally associated with the mitochondria and a network implicated PD and Huntington's disease as those associated with proteins. Molecular networks related to mitochondrial dysfunction and T-cell regulation are hypothesized to underlie the association between dieldrin and PD. These data contribute to a comprehensive transcriptomic and proteomic biomarker framework for pesticide exposures and neurodegenerative diseases.

**Keywords:** pesticides, biomarker, proteomics, systems biology, immune system, mitochondrial dysfunction

## 1. Introduction

The central nervous system (CNS) is a target for chemicals in our environment, and chronic exposures to low doses of pesticides over time can adversely affect human health. The hypothalamus is the primary control center that integrates environmental signals within the endocrine systems, regulating processes such as metabolism (Frankish, et al. 1995), energy balance (Horvath, et al. 2001), circadian rhythms (Saper et al. 2005), and sleep (Machluf et al. 2011), in addition to other physiological processes. As such, this region of the CNS is a sensitive target for neuroendocrine disruption, defined as exogenous substances in the environment that alter normal neuroendocrine function (León-Olea et al. 2014). Neuroendocrine disruptors can elicit effects by agonistic (i.e., stimulatory) or antagonistic (i.e., inhibitory) actions on neurons that produce neuropeptides, neurotransmitters, or neurohormones (Waye & Trudeau, 2011). These exogenous substances include, but are not limited to, pharmaceuticals, personal care products, industrial effluents, and pesticides.

Dieldrin [(1R, 4S, 4aS, 5R, 6R, 7S, 8S, 8aR)-1, 2, 3, 4, 10, 10-hexachloro-1, 4, 4a, 5, 6, 7, 8, 8a-octahydro-6,7- epoxy-1, 4:5, 8-dimethanonaphthalene] (DLD) is an organochlorine legacy pesticide that was used globally as a highly effective insecticide for soil dwelling insects and pests (de Jong, 1991). The pharmacological mode of action of DLD is to antagonize GABA<sub>A</sub> receptors (Ikeda et al. 1998). Despite its effectiveness, dieldrin has been restricted or banned in many parts of the world due to its persistence and adverse effects in non-target species (Martyniuk et al. 2013, Sonne et al. 2014). By 1995, DLD was banned in more than 70 countries (Pesticide Action Network North America, 1995) and in 1997, the Governing Council of the United Nations Environment Programme designated DLD as a persistent organic pollutant (UNEP, 1997, fate reviewed in Jorgenson et al., 2001). However, despite its ban several decades

ago in the US, DLD is still detectable in the environment and has consistently ranked high as a chemical of concern for human health. Most recently, dieldrin was ranked 18<sup>th</sup> on the list of priority chemicals in the 2015 U.S. Agency for Toxic Substances and Disease Registry.

One reason for the concern is that dieldrin has been associated with human neurological disorders such as Parkinson's disease (PD) (Fleming et al., 1994; Corrigan et al., 2000), but direct causative links have remained elusive. In a comparison between a Finnish population and 2001-2002 US National Health and Nutrition Examination Survey population, there was a significant association between plasma levels of DLD and the occurrence of PD (Weisskopf et al. 2010). More recently, Chhillar et al. (2013) detected higher levels of DLD in individuals showing clinical signs for PD in a population in India. In the population, 9.3% of the control subjects with no clinical signs for PD had concentrations of DLD ranging from 0.96 – 4.2 ng/mL in blood while >90% of the control group had no detectable DLD in blood. Conversely, 61.4% of those showing clinical signs for PD also had DLD levels ranging from 2.1 – 28.6 ng/mL. Thus, there is some epidemiological evidence suggesting that elevated DLD in the blood may be associated with an increased risk for PD.

The objectives of this study were to identify molecular networks regulated by DLD in the central nervous system. We elected to conduct our feeding experiment in zebrafish, as this model has become widely adapted for chemical toxicity testing because of their short developmental period (Kimmel et al. 1995), strongly developed genetic tools, well-annotated genome and proteome, and conservation of signaling systems with mammals (Löhr et al. 2011). Zebrafish are used as a model to study human diseases, including neurological disorders (Best & Alderton, 2008; Xi et al. 2011), cardiac disease (Bakkers, 2011), obesity (Tinguad-Sequeira et al. 2011), and cancer (Teittinen et al. 2012). Therefore, we elected to investigate the effects of the

legacy pesticide dieldrin in this species in the hypothalamus, one of the major control centers for integration of neuronal communication and the endocrine system. We also aimed to determine whether dietary DLD affected the behavior of adult zebrafish, as locomotion serves as a phenotypic endpoint for neurological dysfunction and DLD is associated with PD, a movement disorder.

## **2. Methods**

### *2.1. Feed preparation and experimental design*

Twelve milligrams of DLD was dissolved into 4 mL of olive oil and was mixed for 1 hour (stock solution). A serial dilution was performed by adding 0.5 mL of the stock solution into 4.5 mL of fresh oil to generate three treatment feeds (10 times dilution series). Corey Aqua Feed Optimum extruded fish pellets (Corey Feed Mills Ltd., Fredericton, NB, Canada) were coated with either the 4 mL olive oil (control) or one of the three DLD preparations. Experimental feed was prepared by mixing 400 g of pellets in a rotating glass jar and adding 1 mL of each oil treatment every hour (3 mL oil total). Control feed was prepared in the same way but with 3 mL of oil only. The nominal feed concentrations that were prepared were 0, 0.0225, 0.225 and 2.25  $\mu\text{g}$  DLD / grams dry weight (g d.w.) feed to generate a control feed (no DLD), and a low, medium, and high dose feed, respectively. The measured feed concentrations (Section 2.4 describes the analytical methods) were 0, 0.03, 0.15, and 1.8  $\mu\text{g}$  DLD /g d.w. feed. Nominal calculations were based on a study by Muller et al. (2004), which reported that feeding 3  $\mu\text{g}/\text{g}$  to LMB for 30 days resulted in whole body burdens of 0.5  $\mu\text{g}/\text{g}$  wet weight (w.w.). The two lower doses were generated to achieve body burdens of 0.10 – 0.50  $\mu\text{g}/\text{g}$  w.w., levels that approximate concentrations detected in patients with Parkinson's disease (Weisskopf et al.

2010). From this point forward in the manuscript, treatments will be referred to by their measured dose in the feed.

Adult wildtype zebrafish (AB strain, *Danio rerio*, ~5 months of age) were obtained from Mirdo Inc. (Montréal, QC, Canada). Zebrafish were housed at the Canadian Rivers Institute (Saint John, NB, Canada) in 20 L tanks in dechlorinated water. After an acclimation period of 3 weeks, zebrafish were divided into 9 tanks per experimental group giving a total of 36 tanks, each with active carbon in the tank as an extra precaution to minimize any exposure to water-borne DLD. During the study, we did not measure DLD in water as it was unlikely to be detected due to its high hydrophobicity. Studies investigating DLD in water report >95% removal by activated carbon (Bandala et al., 2006).

Five fish were added to each tank, with the goal of maintaining a 3 to 2 female to male sex ratio. However, as zebrafish are difficult to sex externally and this was not the case for every tank. Zebrafish in designated tanks were fed 0.065 g of the control or one of the three contaminated feeds twice a day representing a daily intake of 0.13 g (approximately 4% of fish body weight per tank). Feeding took place at 10 am and 4 pm each day. Every three days, approximately half of the water in the experimental tanks was removed and replaced with fresh water. Feces and uneaten food were removed by siphon daily to minimize exposure to DLD by leaching into the water. Water parameters (mean  $\pm$  standard deviation) measured daily during the experiment were as follows; temperature = 25.5°C ( $\pm$  1.5, n=78), dissolved oxygen = 84.1% ( $\pm$  8.4, n=78), and pH = 6.54 ( $\pm$  0.57, n=78).

After 21 days, two randomly selected zebrafish from each tank were subjected to behavioral testing employing the novel tank test (Section 2.2). Following this, animals were

immediately euthanized in a sodium bicarbonate buffered solution of MS-222 (250 mg/L; Sigma-Aldrich, St. Louis, MO, USA) and their spinal cords were severed. The hypothalami of all male and female zebrafish within a single tank were dissected and pooled into one biological replicate for each sex (i.e. one male and one female pool). Thus, each treatment contained 9 biological replicates for both male and female hypothalami. Females were the focus for all subsequent analyses. The remaining fish carcass was used for DLD measurements in whole body. An animal use protocol for the experiment was carried out ethically in accordance with an approved Animal Care Committee protocol from the University of New Brunswick, Saint John.

## *2.2. Behavioral testing*

The novel tank test was used to assess exploration and locomotion in female zebrafish (Levin et al., 2007; Bencan et al., 2009; Egan et al., 2009; Grossman et al., 2010). Methodology was adapted from Rosemberg et al. (2012). After the 21 day exposure, individual zebrafish were placed in a 1.5 L trapezoidal tank. Two fish per experimental tank were tested for exploratory behavior and activity. These endpoints were defined by time spent in each zone, by the number of crosses out of the bottom portion of the tank, and by the time spent in the top and middle portions of the tank. Zebrafish that explore more readily and enter the top portion of the tank quickly are those that are less anxious in the new environment (Levin et al 2007; Egan et al 2009; Mathur & Guo 2011). The number of female zebrafish assessed using the novel tank test was 11, 12, 10, and 9 for 0, 0.03, 0.15, and 1.8  $\mu\text{g}$  DLD /g treatments.

Tanks were divided into three equal horizontal arenas, marked by a virtual dividing line. Zebrafish swimming behavior was recorded using a SONY Handycam DCR-SR45 for 3 min. at a frame rate of 30 frames/sec. and behavioral parameters (latency to first exit from bottom, time

spent in bottom, time spent in middle, and time to first enter top) were measured using video-tracking software (ANY-maze®, Stoelting CO, USA). All fish were handled carefully to avoid undue stress when moving the animals from the experimental tank to the novel tank. The novel tank was filled with 1.3 L of clean water for each trial. All trials were performed in the same room to ensure uniform illumination and water quality between trials. Statistical analysis was performed using a Kruskal-Wallis Test with treatment as the independent variable (GraphPad Prism® V6). The test statistic is denoted as H with numbers of groups used for comparison indicated. Alpha was set to  $\alpha = 0.05$ .

### *2.3. Measurement of dieldrin in feed and zebrafish carcasses*

The amount of DLD in feed pellets and the whole body burden of DLD in fish were quantified using the same method. Three glass vials for each treatment were filled with whole bodies of zebrafish with hypothalamus removed (about ~8-12 fish per vial) and were taken for DLD measurements (n=3 per treatment). To the best of our knowledge, no study has reported on the measurement of DLD in the brain following a dietary exposure. As zebrafish brains are extremely small, and it was estimated that 30-40 brains would be required to detect DLD, we elected to measure whole fish body burdens.

The tissue was freeze dried for 48 hr., percent moisture was calculated, and the tissue was homogenized using a mortar and pestle. Approximately 2 g of dry zebrafish homogenate (or 10 g of dried feed) was weighed into an accelerated solvent extraction cell (ASE300: Dionex, Sunnyvale, CA, USA) and then filled with Ottawa Sand (Fisher Scientific). The sample was spiked with 100 µL of surrogate containing PCB 209 (AccuStandard, New Haven, CT, USA). Samples were twice extracted using an accelerated solvent extractor (model ASE 300, Dionex,

189 Sunnyvale, CA, USA) with a mixture of 50:50 dichloromethane (DCM)/hexane at 1500 psi and  
190 125°C for 10 min.

191 The extract was concentrated with a Büchi Rotavapor R200 (Büchi Labortechnik AG,  
192 Flawil, Switzerland) and further concentrated with a N-Evap™ 112 nitrogen evaporator  
193 (Organomation Associates Inc., West Berlin, MA, USA) to a volume of 7 mL of 50:50  
194 DCM:hexane. A 1 mL aliquot of the extract was removed and used to gravimetrically determine  
195 the percent extractable lipid. Five mL of the remaining 6 mL was passed through a pre-  
196 calibrated automated gel permeation column (GPC: PrepLinc; J2 Scientific, Columbia, MO,  
197 USA) packed with Bio-Beads S-X3 (J2 Scientific, Columbia, MO, USA). The sample was  
198 eluted through the column with 50:50 DCM:hexane to remove lipids from the sample. The  
199 collected sample was concentrated with a Büchi Rotavapor R200, further concentrated with a N-  
200 Evap™ 112 nitrogen evaporator to 1 mL, and then run through a packed column of 8 g of 1.2 %  
201 deactivated Florisil (Fisher Scientific, Ottawa, ON, Canada) and anhydrous sodium sulfate  
202 (Fisher Scientific, Ottawa, ON). Samples were then eluted with 32 mL of hexane (Fraction 1),  
203 48 mL of 15:85 DCM:hexane (Fraction 2) and 70 mL of 50:50 DCM:hexane (Fraction 3).  
204 Fraction 1 contained the surrogate PCB 209 while Fraction 2 contained DLD. Approximately 1  
205 mL of isooctane (Caledon Laboratory Chemicals, Georgetown, ON, Canada) was added to each  
206 fraction and then the entire fraction was concentrated to a volume of approximately 1 mL. Each  
207 fraction then had 10 µL of pentanitrobenzene (AccuStandard®, Inc, New Haven, CT, USA)  
208 added as an internal standard for internal calibration. Final sample concentrations were adjusted  
209 to compensate for the 1 mL removed for % lipid determination and 1 mL removed from the GPC  
210 clean up.

Individual fractions were analyzed on an Agilent 6890N GC with a <sup>63</sup>Ni Electron capture detector (ECD) (Agilent Technologies, Mississauga, ON, Canada). Exactly 1 µL of sample was injected onto a HP-5 60 m x 0.25 mm x 0.25 µm column (Agilent Technologies, Mississauga, ON, Canada). The internal calibration technique was used for quantifying DLD with pentanitrobenzene as the internal standard (USA EPA Method 8081B). The chromatograms were evaluated and quantified using the Enhanced ChemStation MSD ChemStation version D.03.00.611 (Agilent Technologies, Mississauga, ON, Canada). Method blanks (MBs and method spikes (MSs) were assessed with each batch of 10 samples. The method spikes, which consisted of 10 g of Ottawa sand, were processed in a similar manner to the samples and included 100 µL surrogate containing PCB 209 and 100 µL of DLD standard. Method blanks were comprised of 10 g of Ottawa sand and 100 µL of surrogate only. Percent recoveries for PCB 209 and DLD were determined using certified reference material.

#### *2.4 Microarray analysis*

RNA was extracted using the Qiagen RNeasy® Mini Kit (Qiagen) as per manufacturer's protocol. Samples were assessed for quantity and quality using a Nanodrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE) and 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). The mean (±SD) RNA Integrity Number (RIN) was 9.0 (± 0.7) for female hypothalami pools and samples ranged in RIN values from 7.5 – 9.7.

Microarray analysis was performed on hypothalami collected from females in 0 µg DLD / g (N=5), 0.15 µg / g (N=5), and 1.8 µg / g (N=6) groups; the 0.03 µg/g DLD treatment was not used for microarray analysis as the level of DLD in the fish was not significantly different from control. Microarray processing was performed according to manufacturer's protocols (Agilent Low RNA Input Fluorescent Linear Amplification Kit and Agilent 60-mer oligo microarray

processing protocol, Agilent). The Agilent Zebrafish platform (V3, Catalog ID: G2519F-02647, Agilent) was used to measure the hypothalamic transcriptome and hybridization followed established protocols. Following 17 hours of hybridization, slides were removed from the incubation oven and washed according to the protocol. An ozone-barrier slide cover was placed on each slide prior to scanning. Microarray slides were scanned by Agilent DNA Microarray Scanner with Surescan high-resolution technologies. Raw expression data along with tif images were extracted by Agilent Feature Extraction Software (v10.7.3.1). All microarray data reported in this study follow established guidelines “Minimum Information About a Microarray Experiment (MIAME)” (<http://www.ncbi.nlm.nih.gov/geo/info/MIAME>).

Differentially-expressed genes (DEGs) were identified by first importing raw intensity data into JMP® Genomics (v6). Intensity data were normalized using locally weighted linear regression (LOWESS) with a smoothing factor of 0.2 to account for technical variability across slides (Smyth & Speed, 2003; Berger et al., 2004). The arrays were quality control checked using a distribution analysis that plots the intensity distributions of each microarray slide to ensure these distributions are relatively equal. Normalized intensity data were then filtered based on the limit of detection of the microarrays and any value falling below the limit of detection of 2.08 was assigned a value of 2.08. The limit of detection was determined by calculating the mean of all the dark corners on the microarrays. This accounts for any background signal on the slides. A one-way ANOVA, followed by a multiple test correction using the non-permutation based Benjamini and Hochberg method (Benjamini & Hochberg, 1995) was used to determine which transcripts significantly differed in the hypothalamus. The number of gene probes differentially expressed at both  $p < 0.05$  and those differentially expressed after a post-hoc correction are

reported. All expression data were deposited into Gene Expression Omnibus, an open source repository for transcriptomics data (GSE71638).

A hierarchical cluster analysis was performed using differentially expressed probes ( $p < 0.05$ ) from the 0.15  $\mu\text{g} / \text{g}$  and 1.8  $\mu\text{g} / \text{g}$  treatments. The Fast Ward's method was applied to cluster normalized expression data in JMP v6. The Fast Ward's method calculates the distance between two clusters using ANOVA sum of squares added from all variables. At each generation of the cluster, the within-cluster sum of squares is minimized over all partitions obtainable by merging two clusters from the previous generation. Under the assumptions of multivariate normal mixtures, spherical covariance matrices, and equal sampling probabilities, the Ward's method joins clusters on each level of the hierarchy using maximum likelihood.

#### *2.5. Quantitative Proteomics: Protein Extraction, iTRAQ Labeling and LC-MS/MS*

Total proteins were isolated with 100 mM Tris(hydroxymethyl)aminomethane (Tris)-HCl, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonylfluoride (PMSF) and 0.1% Triton X-100. Proteins were washed in 80% cold acetone to remove impurities. Protein assays were performed using Bradford solution (Invitrogen, Carlsbad, CA, USA) with the SoftMax Pro Software v5.3 (Molecular Devices, Downingtown, PA, USA). Proteins were re-dissolved in denaturant buffer (0.1% SDS (w/v)) and dissolution buffer (0.5 M triethylammonium bicarbonate, pH 8.5) supplied in the iTRAQ Reagents 8-plex kit (AB Sciex Inc., Foster City, CA, USA). For each sample, 60  $\mu\text{g}$  of protein were reduced, alkylated, trypsin-digested, and labeled according to the manufacturer's instructions (AB Sciex Inc.). The hypothalami were labeled with one of the iTRAQ tags (113-116) (control was labelled with 113 and the three treatments were labelled as low = 114, medium = 115, and high dose = 116). This was done for three independent biological replicates/group. Thus, there were 3 iTRAQ experiments conducted ( $n=3$

biological replicates per treatment for each of control, low, medium and high). Fourteen fractions were collected by monitoring the absorbance at 280 nm and area size of each fraction was calculated for % coefficient of variation among three biological replicates (Supplemental Figure S1).

The Q Exactive plus hybrid quadrupole-Orbitrap Mass Spectrometer (ThermoFisher Scientific) was used to collect proteomics data, and offers quadruple precursor ion selection with high-resolution and accurate-mass (HRAM) Orbitrap detection. A search using the National Center for Biotechnology Information (NCBI) Teleostei database (downloaded on Mar. 25, 2015; 729,330 entries) using ProteinPilot v4.5 with the Fraglet and Taglet searches under Paragon<sup>TM</sup> algorithm was conducted. Proteins were quantified as previously described by us (Koh et al., 2012).

Briefly, the accuracy of each protein ratio is given by a calculated error factor from the ProGroup analysis in the software, and a P value is given to assess whether the protein is significantly differentially expressed. The error factor is calculated with 95% confidence error, where it is the weighted standard deviation of the weighted average of log ratios multiplied by Student's t factor. The P value is determined by calculating Student's t factor by dividing the (weighted average of log ratios – log bias) by the weighted standard deviation, allowing the determination of the P value with n) 1 degrees of freedom, where n is the number of peptides contributing to the protein relative quantification (software default settings, AB Sciex, Inc.). To be identified as being significantly differentially expressed, a protein had to contain at least three spectra. An expression change was considered significantly different when the protein showed a fold change cut-off  $> 1.2$  or  $< 0.8$  with  $P < 0.05$  in at least two of three biological replicates, along with a Fisher's combined probability  $< 0.05$  of being different among groups. All data are

present in the Supplemental Files. Further details of the proteomics analysis and search parameters are provided in Cowie et al. (2017).

## 2.6. Bioinformatics

Parametric analysis of gene set enrichment (PAGE) was performed in JMP Genomics 6.0® (Kim & Volsky, 2005). This analysis is used to determine enriched gene ontologies that were associated with the ZF microarray targets. PAGE analysis uses a Z-test of all known gene ontologies and their degree of statistical significance (i.e., a p-value) to identify enriched or over-represented ontologies. A false discovery rate for multiple hypothesis testing was set to  $p = 0.05$  (Benjamini & Hochberg, 1995).

Gene set enrichment analysis was also conducted on both doses, in addition to sub-network enrichment analysis (SNEA). Both analyses were conducted in Pathway Studio 9.0 (Ariadne, Rockville, MD, USA) using the ResNet 9.0, a mammalian database curated by Ariadne (Nikitin et al 2003). SNEA identifies gene networks related to cellular processes or diseases that change with a treatment or disease. SNEA uses known relationships among genes (e.g. relationships based on co-expression patterns, binding, or involvement in common pathways) to build networks focused around gene hubs. These interaction maps are generated using information from the ResNet 9 database. Thus, these are pre-defined molecular networks based on literature (i.e., it is the background or reference group). A total number of 20,902 gene probes for zebrafish were successfully mapped to mammalian homologs using the official gene Name + Alias. For transcriptomics data, SNEA was conducted to identify networks that were significantly affected by DLD. Both cell process and disease sub-networks were queried and

there were 500 permutations of the data to generate the distributions. The entire ZF microarray was used as the background list for the enrichment.

For proteomics, there were 208 proteins that were different in abundance and successfully mapped to Pathway Studios using Name + Alias. Each dose was analyzed separately to identify diseases related to these proteins using Pathway Studio. However, for some networks, proteins that showed a significant change in one or more of the doses were used to build networks, and an average fold change for the protein was obtained across each dose in which the protein was significantly different from control ( $P < 0.05$ ) (i.e., in cases where the protein was differentially expressed in multiple treatments). In 14 cases, the protein showed opposite responses in the HYP (i.e., increasing in the HYP with one dose and decreasing in the HYP following another dose). In these cases, the highest magnitude of response was used to map protein networks. The enrichment p-value for all queries was set at  $p < 0.05$ .

### **3.0. Results**

#### *3.1. Dieldrin in zebrafish whole bodies*

Surrogate recoveries in feed ranged from 81% to 128%, with no correlation to the level of DLD, and MS recoveries were 76% and 99% ( $n=2$ ). DLD recoveries in the certified reference material (CRM 1946 fish tissue National Institute of Standards and Technology, Gaithersburg, MD) were 78% and 125% ( $n=2$ ). The detection limit was assigned to be the lowest point in the calibration curve ( $0.001 \mu\text{g/g}$ ) and this was considered to be the reporting limit (RL). Recovery of surrogate (PCB209) when considering all fish samples was  $78.3 \pm 28.4\%$  ( $n=11$  total), with no correlation to their DLD concentrations. The CRM recovery, method spike, and method blank for the fish samples were 103.2%, 105.1% and 126.9% ( $N=1$  for each), respectively. DLD

recoveries were 78% and 99% for the CRM and MS, respectively. The RL for fish (0.005 $\mu$ g/g) was based upon the lowest point in the calibration curve (0.001  $\mu$ g /g) multiplied by 5, as there was 5 times less tissue per fish sample than in the feed samples. Extractable lipid content in the fish ranged from 27.2 to 33.9 % lipid-dw. Percent moistures for the grouped fish ranged from 66.5 to 70.5%. The wet weight concentrations were calculated to 1.82, 3.50 ( $\pm$  1.75), 17.6 ( $\pm$  4.5) and 148 ( $\pm$  36) ng/g-ww. Dieldrin has been recovered at 88-90% in other studies (Manirakiza et al. 2002; Chen et al. 2009). Thus, the recovery of DLD here is comparable to other studies and extraction protocols.

Control feed contained no detectable DLD and values were set to the RL (0.001  $\mu$ g DLD/g d.w. feed) whereas mean DLD in feed was 0.03, 0.15, 1.8  $\mu$ g DLD/g d.w. feed for the low, medium and high dose, respectively (Figure 1A). The extractable lipid content of the feed ranged 14.1-20.2 % lipid-wet weight (ww) across all doses. Measured concentrations are used when discussing the results. The treatments were significantly different from the control group ( $F_{3,5} = 997.5$ ;  $p < 0.0001$ ) following a Dunnett's post hoc test (Figure 1A).

Dieldrin was also measured in the whole animals. DLD in zebrafish fed the control diet was at the RL  $\sim$ 0.006  $\mu$ g/g dw (n=2) (detection limit was 0.005  $\mu$ g/g dw), and was 0.011 ( $\pm$ 0.005) in the low dose feed (n=3), 0.058 ( $\pm$ 0.016) in the medium dose feed (n=3) and 0.047 ( $\pm$ 0.098) in the high dose feed (n=3)  $\mu$ g DLD/g dry weight (Figure 1B). Lipid based concentrations were: 0.028, 0.034, 0.194 and 1.54  $\mu$ g/g-lipid respectively. The body burden level of DLD in individuals fed low-feed treatment was not significantly different from control animals due to a small sample size but was  $\sim$ 2-fold above reporting limits of the analytical assay (Figure 1B). Both the medium and high dose treatments were different than control fish and there was a significant increase in DLD levels in the zebrafish with dose

(ANOVA,  $F_{(3,8)} = 20.7$ ;  $p < 0.001$ ). The low measured dose was 34 ng DLD /g lipid, which was comparable to 40 ng/g lipid reported in the study by Weisskopf et al. (2010).

### 3.2. Behavioral responses

There were no differences in endpoints that included latency to first exit bottom, time spent in bottom, and time spent in middle of the tank for females (Figure 2A-D). Latency to first exit from the bottom of the novel tank was not different between treatments ( $H_4 = 2.45$ ,  $p = 0.49$ , Figure 2A). The fish spent the majority of time in the bottom of the tank and there were no significant differences between treatments ( $H_4 = 1.24$ ,  $p = 0.74$ , Figure 2B). Time spent in the middle was not significantly different between treatments ( $H_4 = 0.47$ ,  $p = 0.92$ , Figure 2C). The latency to first entering the top portion of the tank was not significantly different between treatments ( $H_2 = 2.47$ ,  $p = 0.48$ , Figure 2D).

### 3.3. Microarray analysis in the hypothalamus

All data for transcripts are reported in Appendix 1. Prior to an FDR correction, there were 2470 probes (2453 unique genes) in the hypothalamus that were differentially expressed in the 0.15  $\mu\text{g/g}$  treatment group when compared to those of the control group ( $p\text{-value} < 0.05$ ). A total of 1240 probes were down-regulated and 1230 probes were up regulated. Some examples of genes that were differentially expressed included solute carrier family 30 (zinc transporter), member 1b (*slc30a1b*, Fold change (FC) = -2.7,  $t = -4.4$ ,  $p < 0.001$ ), and solute carrier family 4, anion exchanger, member 1a (*slc4a1*, FC = 2.8,  $t = 3.7$ ,  $p = 0.002$ ). Transcript gamma-glutamylamine cyclotransferase, tandem duplicate 2 (*ggact*) was significantly down-regulated in the 0.15  $\mu\text{g/g}$  treatment (FC = -11.1;  $t = -2.8$ ,  $p = 0.015$ ) in addition to being affected in the HYP in the 1.8  $\mu\text{g}$  treatment (FC = -6.7;  $t = -2.3$ ;  $p = 0.038$ ). No transcript was significantly altered in

the HYP following an FDR post-hoc correction. This may be due to one or more of the following reasons. (1) High biological variability in the transcriptome, (2) subtle response to a low dose of dieldrin, and (2) a large number of statistical tests performed (more than 40,000). As such, we focus on the pathway analysis to address this concern as it leverages the entire dataset and determines enrichment of processes based upon fold change responses of all the transcripts in a particular biological process.

Prior to an FDR correction, there were 1822 probes (1814 unique genes) in the hypothalamus that were differentially expressed in the 1.8 µg/g treatment group when compared to controls ( $p$ -value  $< 0.05$ ). A total of 1027 probes were down-regulated and 795 probes were up regulated. Some examples of genes that were differentially expressed included solute carrier family 25 (mitochondrial carrier; dicarboxylate transporter), member 10 (*slc25a10*, FC= 2.46;  $t$ = 2.47;  $p$ =0.028) and solute carrier family 6 (neurotransmitter transporter, glycine), member 5 (*slc6a5*, FC= -2.55,  $t$ =-4.08,  $p$ =0.001). No transcript was significantly altered in the HYP following an FDR post-hoc correction.

There were 454 unique probes in common (10.6 %,  $p < 0.05$ ) between the two treatments used in the microarray analysis. When clustering the probes (unadjusted  $p < 0.05$ ), each of the three treatment groups separated out into individual clades in the hierarchical cluster (Supplemental Figure S2).

*3.4 Cell processes related to mitochondrial dysfunction and immunity are preferentially affected by dieldrin at the level of the transcriptome*

Parametric analysis of gene set enrichment analysis (PAGE) was used to identify enriched gene ontology terms (e.g. biological processes, molecular function, and cellular components) in the hypothalamus. In both treatments, there were 14 gene ontologies that were differentially expressed after FDR adjustment and 565 out of 2841 gene sets were differentially expressed before FDR adjustment. In fish from the 0.15 µg/g treatment, a total of 6 gene sets were over-represented including hemoglobin complex, oxygen binding, oxygen transport, oxygen transporter activity, mitochondrial membrane, and intermediate filament (Supplemental Table 1). In fish from the 1.8 µg/g treatment, a total of 8 gene sets were over-represented in the hypothalamus and these included lipid transporter activity, lipid transport, DNA photolyase activity, response to estrogen stimulus, DNA-dependent negative regulation of transcription, signal complex assembly, mitochondrial membrane, and translation elongation factor activity.

Processes related to the mitochondria were affected at the transcript level, based on the PAGE analysis (Supplemental Table S1). Three over-represented gene ontologies that were related to the mitochondria and were altered in both treatments included mitochondrial membrane (GO: 0031966), mitochondrial matrix (GO: 0005759), and adenosine triphosphate (ATP) synthesis coupled electron transport (GO: 0042773). Mitochondrial-related gene ontologies that were identified in the 0.15 µg/g treatment group included the respiratory chain (GO: 0070469), electron transport chain (GO: 0022900), hydrogen ion transporting ATP synthase activity (GO: 0046933), nicotinamide adenine dinucleotide (NADH) dehydrogenase (ubiquinone) activity (GO: 0008137), mitochondrial electron transport, NADH to ubiquinone (GO: 0006120), proton-transporting ATP synthase complex, catalytic core F (GO: 0045261), mitochondrial envelope (GO: 0005740), proton-transporting v-type ATPase, v0 domain (GO: 0033179), mitochondrial iron ion transport (GO: 0048250), and mitochondrial respiratory chain

complex IV (GO: 0005751). Unique gene ontologies identified in the 1.8 µg/g treatment were proton-transporting ATP synthase complex, coupling factor F(o) (GO: 0045263), mitochondrial transport (GO: 0006839), mitochondrial part (GO: 0044429), mitochondrial electron transport, ubiquinol to cytochrome c (GO: 0006122), NADH dehydrogenase activity (GO: 0003954), ATP synthesis coupled proton transport (GO: 0015986), and mitochondrion (GO: 0005739) ( $p < 0.05$ ), suggesting that transcripts related to different functional roles of the mitochondria were impacted by DLD. Between the two treatments, a total of 565 (268 in 0.15 µg/g and 297 in 1.8 µg/g) gene sets were differentially expressed (unadjusted  $p < 0.05$ ) from a total of 2841. There were 41 gene sets that were identified as differentially affected with both doses of DLD, suggesting that these are the processes that are most likely affected by this pesticide. All data for the PAGE analysis are provided in Appendix 2. The subnetwork enrichment analysis also supported the analysis by PAGE and processes related to lactate and pyruvate metabolism, as well as the urea cycle were preferentially affected by dieldrin in the hypothalamus (Supplemental Figure S3).

Gene set enrichment analysis using Pathway Studio revealed that T-cell receptor signaling pathways were preferentially down-regulated in individuals treated with both doses. A total of 8 cell processes related to T-cells were down-regulated in the HYP including lymphocyte proliferation and development (Figure 3, Supplemental Table S2). In fish from the 1.8 µg/g treatment, a total of 9 cell processes related to T-cells were down-regulated in the HYP including T-cell suppression and function. The T-cell processes that were down-regulated in both treatments were T-cell suppression, T lymphocyte proliferation, T-cell response, T-helper lymphocyte response and  $\gamma$ - $\delta$ -T-cell proliferation ( $p < 0.05$ ). Transcripts involved in adaptive immune response, B lymphocyte proliferation, cellular immune response, immunity, and

inflammatory response were also down-regulated (Appendix 3). Noteworthy was that there was a number of interleukin signaling pathways that were down-regulated with both doses, and there were 4 signaling pathways down-regulated in fish from the 0.15 µg/g d.w. treatment related to ILs and 11 signaling pathways down-regulated in fish from the 1.8 µg/g treatment (Supplemental Table 3, Appendix 3). These signaling pathways involved IL2, 4, 7, and 9 among others.

Transcript profiling revealed that there were 183 sub-networks related to diseases that were affected by DLD. A total of 67 disease outcomes were in common between the two treatments. Some diseases that were affected by DLD at the transcriptome level included lymphocyte response, Mycobacterium Infections, Post-thrombotic Syndrome, Common Variable Immunodeficiency, Thalassemia, and Angina (Appendix 3). There was a predominance of immune-related diseases that were affected with both doses. For example, immune diseases - T-helper lymphocyte activity, autoimmune diseases, and T-cell dysfunction - were identified by sub-network enrichment analysis. This further suggests that DLD may be affecting the expression of T-cells in the CNS.

### *3.5. Protein networks related to mitochondrial dysfunction and Parkinson's disease are perturbed by dieldrin*

A total of 3,941 proteins were identified in the hypothalamus. Figure 4 shows a Venn diagram of the overlap of proteins across the three iTRAQ experiments, proteins that showed 95% confidence in assignments. There were 515 proteins that were identified in all three iTRAQ experiments. Using a fold change cut off of <0.8 or >1.2-fold, there were 226 proteins that were different in abundance in one or more DLD treatments ( $p < 0.05$ ). The number of differentially expressed proteins that were affected by DLD based on dose was as follows: low = 99, middle = 112, and high = 167. Notable up-regulated proteins in the low treatment included those related

to mitochondria such as NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 12 (FC = -3.69; p=0.048), NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 6 (FC=-2.08; p=0.004) and ATP synthase subunit alpha, mitochondrial (FC= -1.56; p=0.014) was down regulated. In the medium treatment ATP synthase subunit gamma, mitochondrial (FC= -2.86; p=0.003) and pyruvate dehydrogenase E1 alpha 1 (FC= 2.38; p= 0.002) and Fructose-bisphosphate aldolase B (FC= 1.75; p= 0.009) were up-regulated in fish from the high treatment as well as NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 6 (FC= 2.11; p= 0.004).

Table 1 lists the proteins that showed a significant change in all three DLD doses, and these are those proteins most likely regulated by DLD. Many were involved in mitochondrial function and bioenergetics (2-methoxy-6-polyprenyl-1,4-benzoquinol methylase (mitochondrial), NADH dehydrogenase-related proteins, malate dehydrogenase, pyruvate dehydrogenase, and ATP subunits). Other notable proteins that were increased by DLD in all three doses included AKT-interacting protein (upregulated 50-90 fold), signal recognition particle 9 (upregulated 30-40 fold), PREDICTED: programmed cell death 6 interacting protein isoform X3 (upregulated 2-fold), and tumor suppressor p53-binding protein 1 (upregulated 40-fold). Proteins that were decreased by DLD in all three doses included ATP synthase subunit alpha, mitochondrial (down-regulated 1.3-2 fold) and Mitochondrial tRNA-specific 2-thiouridylase 1 (down-regulated 2-50 fold). A subnetwork enrichment analysis using differentially expressed proteins also supports the hypothesis that DLD alters the abundance of proteins involved in oxidative stress (p=0.0017), the respiratory chain (p=0.0019), and those related to mitochondrial damage (p=0.003) (Figure 5).

Other major processes impacted by DLD included actin organization, microtubule cytoskeleton assembly, translation, and endocytosis. Diseases were also queried to determine

which ones were associated to the regulated proteins (Table 2). Diseases identified by SNEA and related to the central nervous system were neurodegenerative diseases (PD and Huntington's disease) and neuron viability (Figure 6). All disease-protein networks were higher in abundance based upon the protein levels, except for neuron viability, muscular diseases, and neoplasms in the high DLD group. Noteworthy was that both the low and medium dose of DLD resulted in significant changes in proteins related to PD and this network was increasing in expression ~2-fold. Figure 6 shows some diseases that are related to altered proteins in fish from the medium dose (0.15 µg/g). Proteins in this network that were related to PD included Ubiquitin C-Terminal Hydrolase L1, Cysteine Rich Protein 2, Ataxin 3, Microtubule Associated Protein Tau, Septin 5 and Glyceraldehyde-3-Phosphate Dehydrogenase.

#### **4. Discussion**

##### *4.1 Body burden levels of dieldrin in zebrafish*

Zebrafish incorporated DLD into their tissues over a relatively short feeding regime. The rapid accumulation of DLD was comparable to that observed in other fish species in laboratory experiments. Largemouth bass fed ~3.0 µg DLD /g feed for 2 months had 0.036 µg DLD /g w.w. in muscle (1-2% of the total feed concentration) (Martyniuk et al. 2010a). In another laboratory study, LMB accumulated ~40 % of the total DLD fed to the fish after 30 or 50 days of exposure (Muller et al. 2004). Wild fish also accumulate DLD. In herring (*Clupea harengus*) sampled in the northern part of the Baltic Sea, concentrations of DLD were 0.121 µg/g lipid (Strandberg et al 1998). Thus, our target body concentrations for DLD were within the range of environmentally-relevant levels found in fish species.

In addition to targeting relevant levels for wild fish, we aimed to achieve levels that were relevant to human health. The lower doses were generated to produce a level that approximates concentrations detected in patients with PD (Weisskopf et al. 2010). The low measured dose here was 34 ng DLD /g lipid, which was comparable to 40 ng/g lipid reported in the study by Weisskopf et al. (2010). This is important for human health as fish advisories can exist for high levels of organochlorine pesticides (OCPs), and trophic transfer of these chemicals through the food web and diet are the most likely exposure scenario for humans.

#### *4.2. Behavioral Changes in Zebrafish Exposed Neuroactive Chemicals*

Behavior is an important phenotype for studying the relationship between neuroactive chemicals and neurological conditions such as anxiety and neural activity. Adult female zebrafish fed DLD over 21 days did not show any changes related to exploratory behavior and anxiety. A lack of an effect may be due to the low dose and the relatively short exposure duration, and questions remain about longer term exposures to DLD and behavior. To the best of our knowledge, there are no studies that explore the relationship between body burden levels of organochlorine pesticides such as DLD and behaviors in fish. However there is some evidence that DLD disrupts complex prey-predator behaviors in birds. Loggerhead Shrikes fed 1 mg DLD/kg body weight until they were 25 days old showed prolonged mouse killing behavior compared to the control group (Busbee, 1977). Moreover, lindane, a related organochlorine pesticide to dieldrin that also inhibits GABA<sub>A</sub> receptor signaling, affected behaviors related to exploration and anxiety in male Wistar rats. This included a reduction in the number of total arm and open arm entries in a plus maze at a dose of 20 mg/kg. These GABA receptor-acting organochlorine pesticides can affect behavioral responses and patterns in animals.

It should be pointed out that in zebrafish, some model compounds do not always result in conserved molecular and organismal responses compared to their mammalian counterparts due to different anatomical organization of the CNS. For example, studies with larval zebrafish and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) have revealed that different populations of DA neurons are affected compared to mammals, and this may underlie locomotor behavioral changes observed in some studies (Bretaud et al., 2004; Lam et al., 2005; McKinley et al., 2005; Sallinen et al., 2009a,b).

#### *4.3 Dieldrin preferentially disrupted gene networks related to the mitochondria*

Transcriptomic and proteomic data suggested that DLD affected gene networks related to mitochondrial bioenergetics and oxidative respiration in the hypothalamus. Transcripts related to the mitochondria such as *ND4*, *ND1*, *sdhaf2*, *cox16*, and *cox7a1*, and gene ontologies such as “mitochondrial membrane mitochondrial matrix” and “ATP synthesis coupled electron transport” were preferentially over-represented in the transcriptomic data, suggesting that DLD exposure affects mitochondria function at the transcript level. Proteins altered by DLD included ATP subunits and protein network analysis showed that proteins associated with the respiratory chain and oxidative stress were differentially expressed. These data are consistent with one reported mode of action for DLD; inhibition of complex III of the mitochondrial electron transport system (Bergen, 1971). Our data here are also consistent with feeding studies in LMB, in which proteins related to mitochondria and mitochondrial intermembrane protein transporter complex in the hypothalamus were significantly affected after DLD treatments (Martyniuk et al. 2010a).

Transcripts related to ATP synthase mitochondrial complexes in the LMB hypothalamus increased in steady state mRNA levels following dietary exposure to DLD. In the mitochondria

of the mosquitofish (*Gambusia affinis*), DLD decreased the activity of succinic dehydrogenase (Moffett & Yarbrough 1972), also known as the respiratory Complex II enzyme involved in cellular respiration. Additional *in vitro* studies support the hypothesis that DLD induces mitochondrial dysfunction in the CNS. In rat dopaminergic neural (N27) cells treated with DLD, there was a rapid increase in reactive oxygen species (ROS), followed by a release of cytochrome c into the cytosol (Kanthasamy et al. 2008). Dieldrin may impair mitochondrial function irreversibly by arresting the flow of electrons at or near cytochrome b via NADH dehydrogenase and succinate dehydrogenase, thus lowering cellular ATP production (Bergen, 1971). Altered transcript abundance for genes involved in the electron transport chain support that hypothesis that DLD disrupts the mitochondrial membrane function. Studies should test the hypothesis that mitochondrial bioenergetics are disrupted *in vivo* following dietary DLD exposure. A concern for human health is that many neuroactive pesticides include mitochondrial dysfunction, and this is one mechanism by which neuroactive pesticides can exacerbate human diseases.

#### 4.4. Dieldrin affects T-cell signaling cascades at the transcript level in the hypothalamus

Gene networks related to T-cell receptor pathways and interleukins were suppressed following dietary exposure to DLD, suggesting that DLD has immunotoxic properties. Laboratory studies in general support the hypothesis that DLD induces both an oxidative stress response in tissues and disrupts the immune system in vertebrates, from humans to fish. For example, DLD exposure resulted in the production of reactive oxygen species *in vitro* in primary human neutrophils at 10 and 50  $\mu\text{M}$ , in addition to increasing interleukin 8 production 300% (Pelletier et al. 2001). In mice (BALB/c (Bagg Albino), a 10-week exposure to 1 or 5  $\mu\text{g}$  DLD /g showed higher levels of mortality when challenged with malaria or Leishmania compared to

control mice, suggesting that the mice were immunocompromised (Loose, 1982). In rats exposed to low-moderate doses of DLD in a mixture of OCPs for 70 days via oral gavage, there was impaired liver physiology and T cell function (Wade, et al. 2002). Dieldrin also suppressed the primary IgM response to thymo-dependent and T cell-independent antigens in primary sheep blood culture (Bernier et al. 1987). In non-mammalian vertebrates, DLD has been linked to immunosuppression in juvenile leopard frogs (*Rana pipiens*) by increasing susceptibility to parasitic nematode infection (Christin et al. 2012) and in largemouth bass, Martyniuk et al. (2010b) demonstrated that DLD induced transcripts such as coagulation factor XI (f11), toll-interleukin-1 receptor interacting protein II (tlr2), alpha-2-macroglobulin (a2m), and small inducible cytokine subfamily E member 1 (scye1) in the hypothalamus. Thus, there can be a range of effects on both the innate and adaptive immune systems and additional studies focused on functional endpoints of the immune system will improve understanding regarding the mechanisms underlying pesticide-mediated immunosuppression. Epidemiological and laboratory-based studies provide evidence for compromised immune systems following DLD treatments and OCPs appear to act as immunosuppressive compounds in multiple taxa, including fish (Martyniuk et al. 2016).

#### *4.5. Relevance of dieldrin to Parkinson's disease?*

In terms of the pathways and major themes identified as being perturbed by DLD, there was congruence of both transcriptomic and proteomic data on mitochondria and the immune system. The mitochondrion is considered to be a critical organelle that controls cell death in many neurodegenerative disorders, including PD (reviewed in Golpich et al., 2017). Other mitochondrial respiration inhibitors including 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), paraquat, and rotenone are well documented to be associated with Parkinson-like

symptoms (Langston & Ballard, 1984, Tieu, 2011). We hypothesize that DLD either directly or indirectly affects mitochondrial bioenergetics in dopaminergic neurons, and this results in compensatory molecular responses in the tissue to improve or maintain oxidative phosphorylation. The immune system also plays a significant role in the etiology of PD and our data suggest that T-cell dysfunction is related to DLD-induced neurotoxicity. Chen and colleagues (2016) recently summarize data in a review that shows that microglia and astrocytes lead to immune dysregulation and inflammation in the CNS in PD. With DLD exposure, suppressed transcriptional expression of the immune system observed here may act to mitigate or reduce neuro-inflammation. There also appears to be a close relationship between mitochondrial pyruvate metabolism, inflammation, and PD (Ghosh et al. 2016) and DLD and other pesticides appear to modulate these types of interactions, leading to PD pathogenesis.

A link between DLD, mitochondria, the immune system, and PD is strengthened by the pathway analysis of disease-related processes for proteins in this study and many biomarkers for neurodegeneration were observed here. For example, cathepsin D, has been linked to neurodegenerative disorders in humans (Steinfeld et al. 2006) and was down-regulated with DLD treatment. Microtubule-associated protein tau was also down-regulated, and mutations in this gene are associated with hereditary frontotemporal dementia and Parkinsonism-linked chromosome 17 (Satake, et al. 2009). In another proteomics study that investigated the effects of DLD on the hypothalamic proteome in largemouth bass, Martyniuk et al. (2010a) reported a significant increase in ATP synthase, H<sup>+</sup> transporting, mitochondrial F1 complex, beta polypeptide (ATP5b), ATP synthase, H<sup>+</sup> transporting, mitochondrial F1 complex alpha subunit 1, cardiac muscle (ATP5a1), and cytosolic cytochrome (CytC), thus there may be conserved proteomic responses to dieldrin in the hypothalamus of other fish. Overall, our dataset contained

52 proteins affected by DLD that were also reported to be differentially altered in abundance in at least one proteomics experiment in the CNS of Parkinson's patients (Werner et al 2008; Basso et al., 2004; Jin et al. 2006; Licker et al. 2014; Chen et al. 2012). Some of these proteins included ATP synthase D chain (mitochondrial), glyceraldehyde-3-phosphate dehydrogenase, and ubiquitin carboxyl-terminal esterase L1, proteins involved in oxidative respiration and protein degradation. The zebrafish has become a useful Parkinson's disease model for protein biomarker discovery for PD-inducing pharmaceuticals such as MPTP (Sarath Babu et al., 2016) and studies such as this lay the foundation for a comprehensive proteomic and transcriptomic biomarker framework for pesticide exposures and risk to neurodegenerative diseases.

## Acknowledgments and funding

The authors have no conflict of interest to declare. We would like to thank Dr. H. Taukilus for providing the behavior software. This research was funded by a New Brunswick Research Assistantship Initiative grant from the New Brunswick Innovation Foundation (RAI 2015-080, CJM), Canada Research Chair program (CJM, KAK), and Natural Sciences and Engineering Research Council of Canada Discovery Grants (386275-2010, CJM; 4293899-2012, KAK).

## Figure Captions

**Figure 1.** (A) Dieldrin (DLD) concentration in fish pellets ( $\mu\text{g DLD/g food (dry weight)}$ ) in control and the three doses of DLD. Each treatment group was different from controls ( $p < 0.0001$ ,  $N = 2-3$  per group). Dieldrin was below the reporting limit (RL) in control pellets ( $\text{RL} = 0.001 \mu\text{g/g}$ ). (B) Concentrations of DLD in zebrafish after 21 days of feeding ( $N = 2-3$  per group). Body burdens of zebrafish in the lowest treatment were not different from controls whereas carcasses from the medium ( $p < 0.05$ ) and highest ( $p < 0.001$ ) dose were different from control fish. The reporting limit for dieldrin was  $0.005 \mu\text{g/g}$ .  $p < 0.0001 = ***$ ;  $p < 0.001 = **$ ;  $p < 0.05 = *$ . Data are presented as  $\log_{10}$  transformed values.

**Figure 2.** The behavior of female zebrafish did not differ by treatment for latency to first exit bottom (A), time spent in bottom (B), time spent in middle (C), and latency to first entering into the top portion of the tank (D). Data are presented as mean  $\pm$  SEM.

**Figure 3.** A transcriptome network for cell processes related to T-cells following a measured dose of 1.8  $\mu\text{g}$  DLD /g d.w. feed. Red indicates that the transcript is increased in abundance and blue indicates that transcript is decreased in relative abundance compared to controls. Abbreviation are provided in Appendix 4.

**Figure 4.** Venn diagram showing overlap between protein identification in three iTRAQ experiments (Set 1, Set 2, and Set 3). The bottom table summarizes the conclusions of the proteomics analysis “experiment-wide”. A total of 3, 556 proteins were quantified with at least three unique spectra based on peak intensities of iTRAQ labeling tags, coupled with normalization and bias correction as previously described in Koh *et al.* (2012). An expression change was considered significantly different when the protein showed a fold change cut-off  $> 1.2$  or  $< 0.8$  with  $P < 0.05$  in at least two of three biological replicates, along with a Fisher’s combined probability  $< 0.05$  of being different among groups.

**Figure 5.** A protein network for cell processes in the HYP following exposure to dieldrin. Red indicates that the protein is increased in abundance and blue indicates that protein is decreased in relative abundance. Proteins related to respiratory chain, mitochondrial damage and oxidative stress were significantly affected by dieldrin compared to control. Abbreviation are provided in Appendix 4.

**Figure 6.** A protein network for diseases following a measured low dose of 0.03  $\mu\text{g}$  DLD /g d.w. feed. Red indicates that the protein is increased in abundance and blue indicates that protein is decreased in relative abundance. Abbreviation are provided in Appendix 4.

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**Table 1** Proteins which were significantly altered at all three doses of dieldrin. Presented are the proteins (accession and abbreviation), % coverage of the proteins by peptides, number of peptides used in the quantitation in the iTRAQ experiment, fold change for the three doses (low, medium, and high), and p-value (p<0.05). FC = fold change.

| <i>Name</i>                                                     | <i>Accession</i> | <i>%Cov</i> | <i>Fc<br/>low</i> | <i>p-value<br/>low</i> | <i>Fc<br/>medium</i> | <i>p-value<br/>medium</i> | <i>Fc<br/>high</i> | <i>p-value<br/>high</i> |
|-----------------------------------------------------------------|------------------|-------------|-------------------|------------------------|----------------------|---------------------------|--------------------|-------------------------|
| 2-methoxy-6-polyprenyl-1,4-benzoquinol methylase, mitochondrial | gi 82234518      | 14.7        | 5.12              | 0.037                  | 5.3                  | 0.036                     | 3.99               | 0.022                   |
| 60S acidic ribosomal protein P2                                 | gi 154426308     | 79.8        | 0.91              | 0.041                  | 0.8                  | 0.028                     | 1.66               | 0.021                   |
| AKT-interacting protein                                         | gi 82209840      | 13.3        | 93.49             | 0.025                  | 50.04                | 0.025                     | 93.49              | 0.024                   |
| ataxin-3                                                        | gi 63053592      | 16.7        | 2.03              | 0.037                  | 1.92                 | 0.024                     | 2.01               | 0.025                   |
| ATP synthase subunit alpha, mitochondrial                       | gi 116325975     | 63.5        | 0.64              | 0.014                  | 0.8                  | 0.01                      | 0.67               | 0.016                   |
| dipeptidyl peptidase 3                                          | gi 50540434      | 19.3        | 1.88              | 0.005                  | 2.22                 | 0.005                     | 0.5                | 0.018                   |
| glutaminyl-tRNA synthetase                                      | gi 49619001      | 13.1        | 1.6               | 0.021                  | 1.22                 | 0.025                     | 1.45               | 0.002                   |
| high-mobility group nucleosome binding domain 1-like            | gi 238550145     | 43          | 1.9               | 0.002                  | 2.56                 | 0.004                     | 1.29               | 0.001                   |
| Histone H2AX                                                    | gi 73919733      | 73.9        | 1.25              | 0.003                  | 1.29                 | 0.002                     | 1.04               | 0.011                   |
| Mitochondrial tRNA-specific 2-thiouridylase 1                   | gi 82192768      | 10.8        | 0.32              | 0.026                  | 0.84                 | 0.024                     | 0.01               | 0.003                   |
| Myb-binding protein 1A-like protein                             | gi 71153824      | 11.2        | 3.72              | 0.037                  | 3.75                 | 0.017                     | 3.07               | 0.031                   |
| NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 12          | gi 74315939      | 34.9        | 3.69              | 0.048                  | 3.72                 | 0.045                     | 3.84               | 0.045                   |
| NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 6     | gi 41387136      | 39.8        | 2.08              | 0.004                  | 2.17                 | 0.004                     | 2.11               | 0.004                   |
| N-alpha-acetyltransferase 30, NatC catalytic subunit            | gi 348604841     | 10.2        | 5.46              | 0.034                  | 4.82                 | 0.032                     | 4.65               | 0.034                   |
| neurofilament medium polypeptide isoform 1                      | gi 162287417     | 49          | 1.16              | P<0.001                | 2.52                 | P<0.001                   | 1.07               | 0.005                   |
| non-muscle caldesmon                                            | gi 168823552     | 32.2        | 0.72              | 0.022                  | 0.96                 | 0.008                     | 1.81               | 0.002                   |
| PREDICTED: ataxin 2-like isoform X1                             | gi 528473441     | 9.3         | 5.6               | 0.012                  | 8.21                 | 0.015                     | 3.83               | 0.021                   |
| PREDICTED: chromosomal protein D1 isoform 2                     | gi 68391583      | 28.6        | 1.8               | 0.002                  | 1.4                  | 0.002                     | 1.07               | 0.009                   |
| PREDICTED: collagen, type I, alpha 1b isoform X4                | gi 528495236     | 26.7        | 1.46              | 0.022                  | 1.45                 | 0.023                     | 2.28               | 0.002                   |
| PREDICTED: cytoplasmic FMR1-interacting protein 2 isoform X1    | gi 528500134     | 14.7        | 1.56              | 0.002                  | 1.56                 | 0.002                     | 1.49               | 0.002                   |
| PREDICTED: filamin-B                                            | gi 528494954     | 16.4        | 3.35              | 0.003                  | 1.69                 | 0.004                     | 1.88               | 0.003                   |
| PREDICTED: histone H2B 1/2-like                                 | gi 68362792      | 75          | 2.21              | 0.023                  | 1.6                  | 0.02                      | 1.37               | 0.03                    |
| PREDICTED: keratin 12 isoform X1                                | gi 528509044     | 40          | 2.81              | 0.001                  | 1.9                  | 0.009                     | 1.26               | 0.009                   |
| PREDICTED: malate dehydrogenase 1a, NAD (soluble) isoform X2    | gi 528469134     | 49.8        | 0.7               | 0.003                  | 0.64                 | 0.003                     | 1.05               | 0.048                   |
| PREDICTED: neurofilament light polypeptide-like                 | gi 189533895     | 40.8        | 0.46              | 0.006                  | 3.43                 | P<0.001                   | 0.9                | 0.001                   |

|                                                                                          |              |      |       |       |       |       |       |       |
|------------------------------------------------------------------------------------------|--------------|------|-------|-------|-------|-------|-------|-------|
| PREDICTED: non-histone chromosomal protein HMG-14A                                       | gi 528501164 | 37.6 | 3.2   | 0.003 | 4.85  | 0.002 | 2.06  | 0.004 |
| PREDICTED: programmed cell death 6 interacting protein isoform X3                        | gi 528510445 | 14.1 | 2.52  | 0.026 | 2.4   | 0.021 | 1.86  | 0.035 |
| PREDICTED: proline-, glutamic acid- and leucine-rich protein 1-like                      | gi 292616020 | 9.2  | 1.57  | 0.004 | 3.53  | 0.004 | 2.18  | 0.002 |
| PREDICTED: protein LYRIC isoform X3                                                      | gi 528510123 | 19.9 | 1.48  | 0.003 | 1.14  | 0.005 | 1.1   | 0     |
| PREDICTED: protocadherin-7-like isoform X2                                               | gi 528467687 | 16.9 | 0.8   | 0.023 | 12.35 | 0.039 | 0.82  | 0.045 |
| PREDICTED: septin-6 isoform X4                                                           | gi 528500155 | 39.1 | 5.46  | 0.035 | 5.32  | 0.028 | 5.38  | 0.022 |
| PREDICTED: serine/threonine-protein kinase DCLK2-like                                    | gi 528518994 | 16.1 | 0.51  | 0.047 | 44.43 | 0.023 | 0.51  | 0.047 |
| PREDICTED: SH3 domain-containing kinase-binding protein 1 isoform X1                     | gi 528519988 | 15.9 | 3.04  | 0.038 | 4.41  | 0.019 | 2.25  | 0.031 |
| PREDICTED: solute carrier family 12 member 4 isoform X1                                  | gi 528485488 | 11.9 | 50.24 | 0.004 | 50.18 | 0.004 | 50.19 | 0.004 |
| PREDICTED: talin-2 isoform X2                                                            | gi 528485133 | 16.5 | 1.46  | 0.01  | 1.67  | 0.003 | 2.53  | 0.003 |
| PREDICTED: uncharacterized protein LOC100002920 isoform X1                               | gi 528479036 | 10.5 | 50    | 0.021 | 49.55 | 0.049 | 49.55 | 0.001 |
| PREDICTED: wu:fd55e03 isoform X1                                                         | gi 528485105 | 24.5 | 0.45  | 0.003 | 0.6   | 0.003 | 0.37  | 0.008 |
| Proteasome (prosome, macropain) 26S subunit, non-ATPase, 13                              | gi 42542524  | 15.9 | 0.77  | 0.017 | 0.43  | 0.015 | 1.61  | 0.011 |
| pyruvate dehydrogenase E1 alpha 1                                                        | gi 53749653  | 32.3 | 2.68  | 0.004 | 1.07  | 0.005 | 2.38  | 0.002 |
| ribosome binding protein 1 homolog 180kDa                                                | gi 37681927  | 39.3 | 1.55  | 0.005 | 1.07  | 0     | 1.09  | 0.023 |
| signal recognition particle 9                                                            | gi 41055367  | 25.6 | 34.63 | 0.001 | 46.02 | 0.002 | 42.33 | 0.002 |
| Tat protein                                                                              | gi 182889742 | 12.4 | 3.13  | 0.003 | 3.04  | 0.001 | 8.68  | 0.002 |
| tumor suppressor p53-binding protein 1                                                   | gi 122114547 | 10.4 | 44.5  | 0.02  | 35.34 | 0.02  | 41.49 | 0.016 |
| tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide | gi 41152453  | 64.1 | 1.48  | 0.002 | 0.94  | 0.002 | 1.42  | 0.003 |
| uncharacterized protein LOC100141350 precursor                                           | gi 168823510 | 30.9 | 1.84  | 0.003 | 2.45  | 0.006 | 1.38  | 0.011 |
| Zgc:65851                                                                                | gi 41944583  | 58.5 | 0.53  | 0.027 | 1.13  | 0.006 | 0.51  | 0.002 |

**Table 2**

**Table 2:** Sub-network enrichment analysis for differentially expressed protein and diseases. Disease are organized by *p*-value within each treatment. Low = 0.03, Medium = 0.15, and High = 1.8 µg/g DLD in pelleted feed.

| <i>Dieldrin</i><br>(µg/g) | <i>Gene Set Seed</i> | <i># of Measured<br/>Neighbors</i> | <i>Median<br/>change</i> | <i>p-value</i> |
|---------------------------|----------------------|------------------------------------|--------------------------|----------------|
| 0.03                      | Neuron viability     | 5                                  | 1.55                     | 0.014          |
| 0.03                      | Hypersensitivity     | 9                                  | 1.90                     | 0.028          |
| 0.03                      | Parkinson Disease    | 8                                  | 1.84                     | 0.045          |
| 0.03                      | Acute-Phase Reaction | 9                                  | 1.84                     | 0.047          |
| 0.15                      | Hypersensitivity     | 9                                  | 3.09                     | 0.019          |
| 0.15                      | Anaphylaxis          | 6                                  | 2.45                     | 0.023          |
| 0.15                      | Acute-Phase Reaction | 9                                  | 2.45                     | 0.025          |
| 0.15                      | Huntington Disease   | 6                                  | 1.92                     | 0.026          |
| 0.15                      | Proteinuria          | 7                                  | 2.45                     | 0.036          |
| 0.15                      | Tuberculosis         | 7                                  | 1.45                     | 0.041          |
| 0.15                      | Parkinson Disease    | 8                                  | 1.92                     | 0.049          |
| 1.8                       | Breast Neoplasms     | 20                                 | 1.10                     | 0.008          |
| 1.8                       | Neuron viability     | 5                                  | -1.78                    | 0.009          |
| 1.8                       | Muscular Diseases    | 7                                  | -1.16                    | 0.011          |
| 1.8                       | Neoplasms            | 72                                 | -1.11                    | 0.023          |
| 1.8                       | Body weight          | 8                                  | 1.65                     | 0.029          |

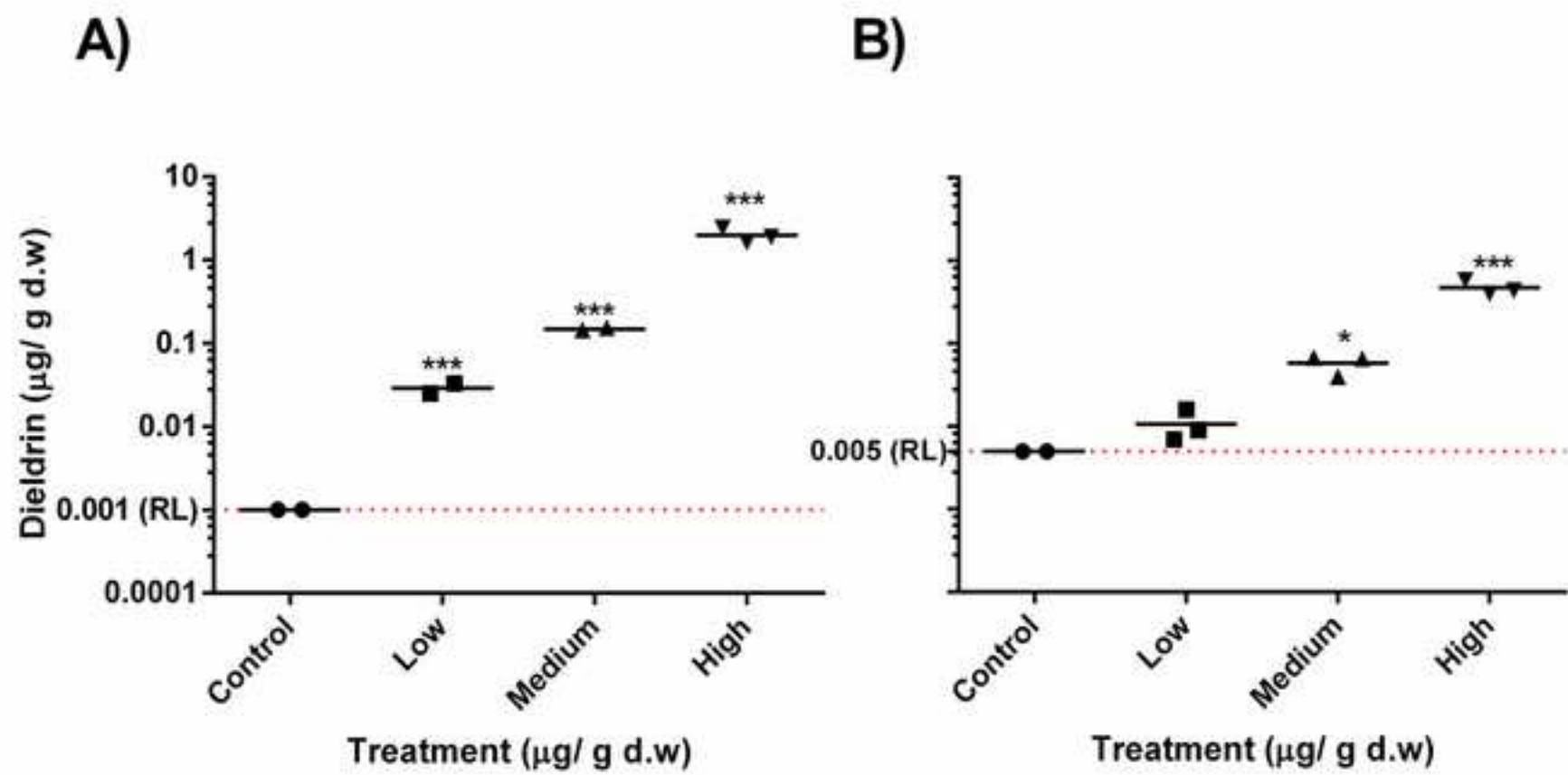
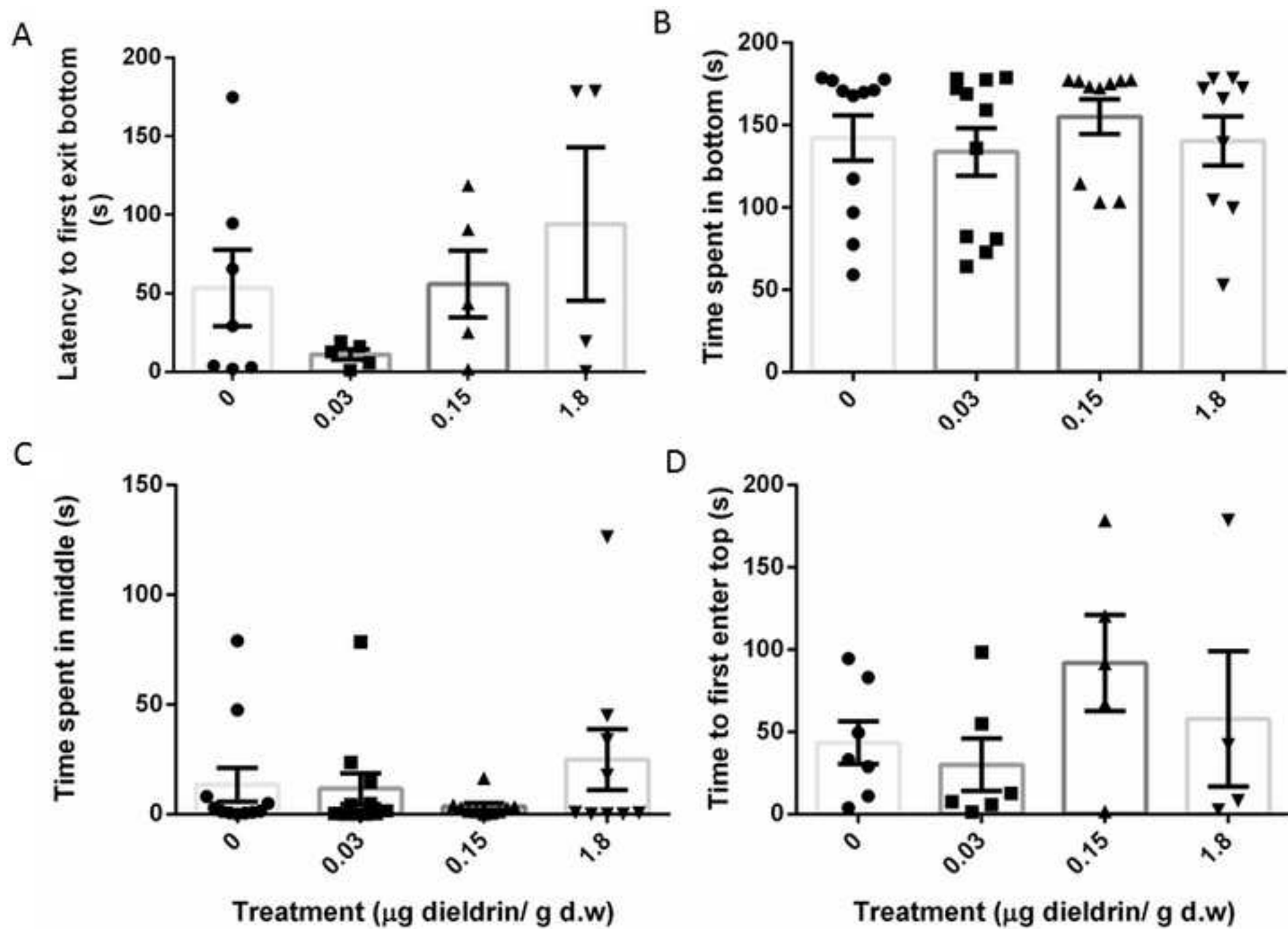


figure 2



### figure 3

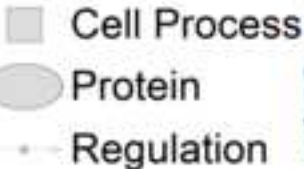
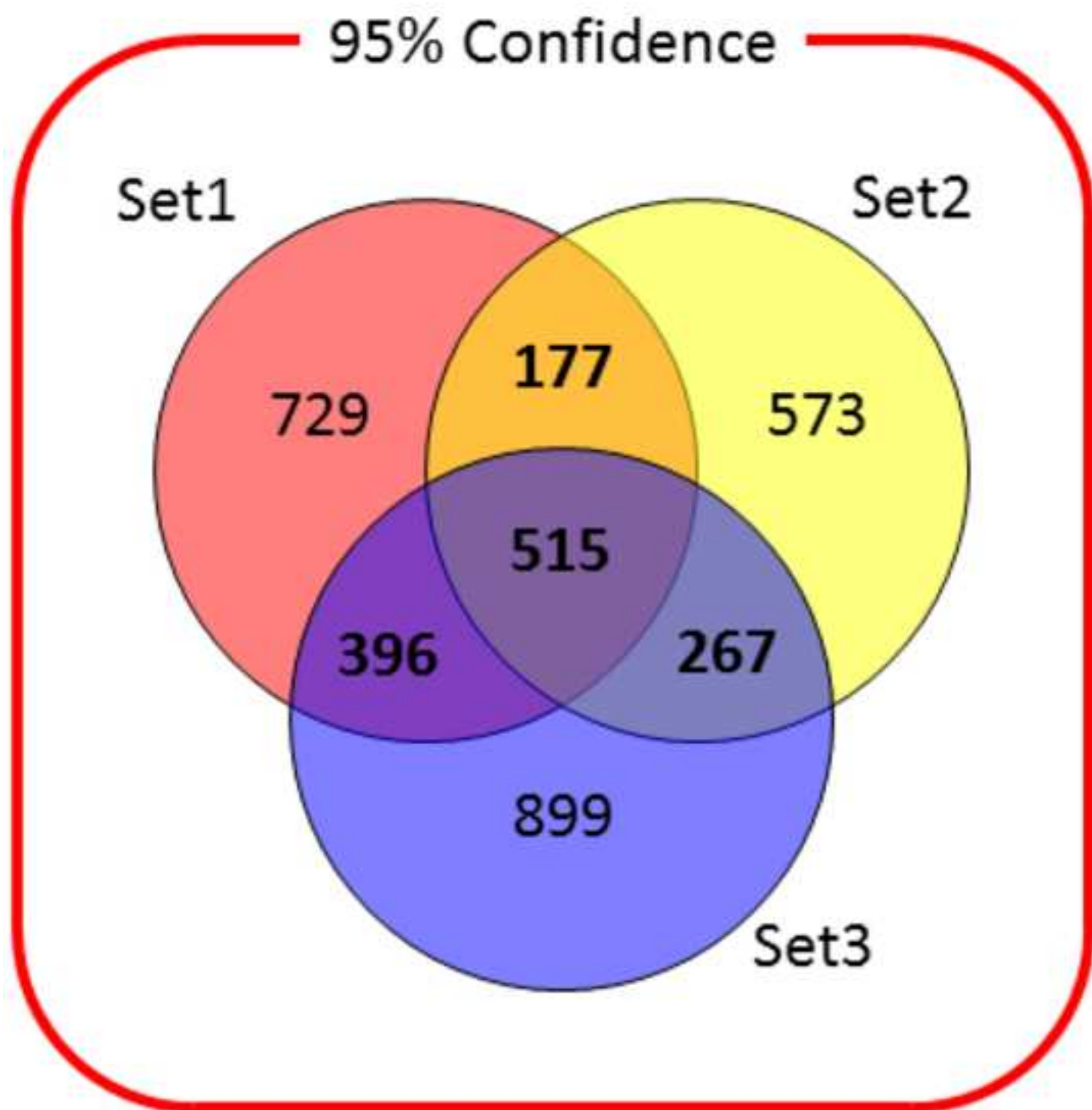


figure 4



| FC Cut-off with P val<0.05 | Differentially Expressed Proteins |
|----------------------------|-----------------------------------|
| 0.8> or >1.2               | 226                               |
| 0.5> or >1.5               | 147                               |

figure 5

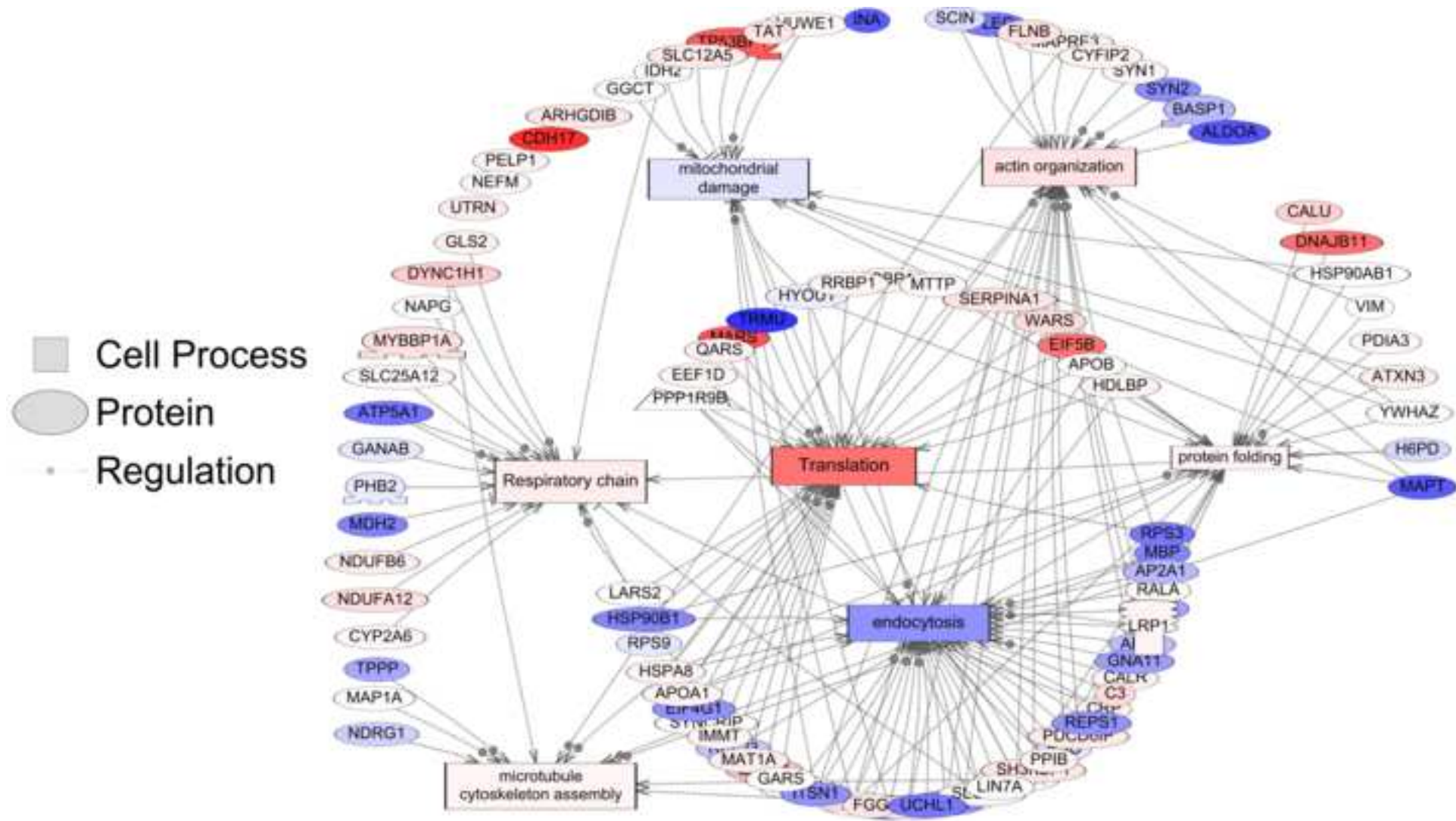
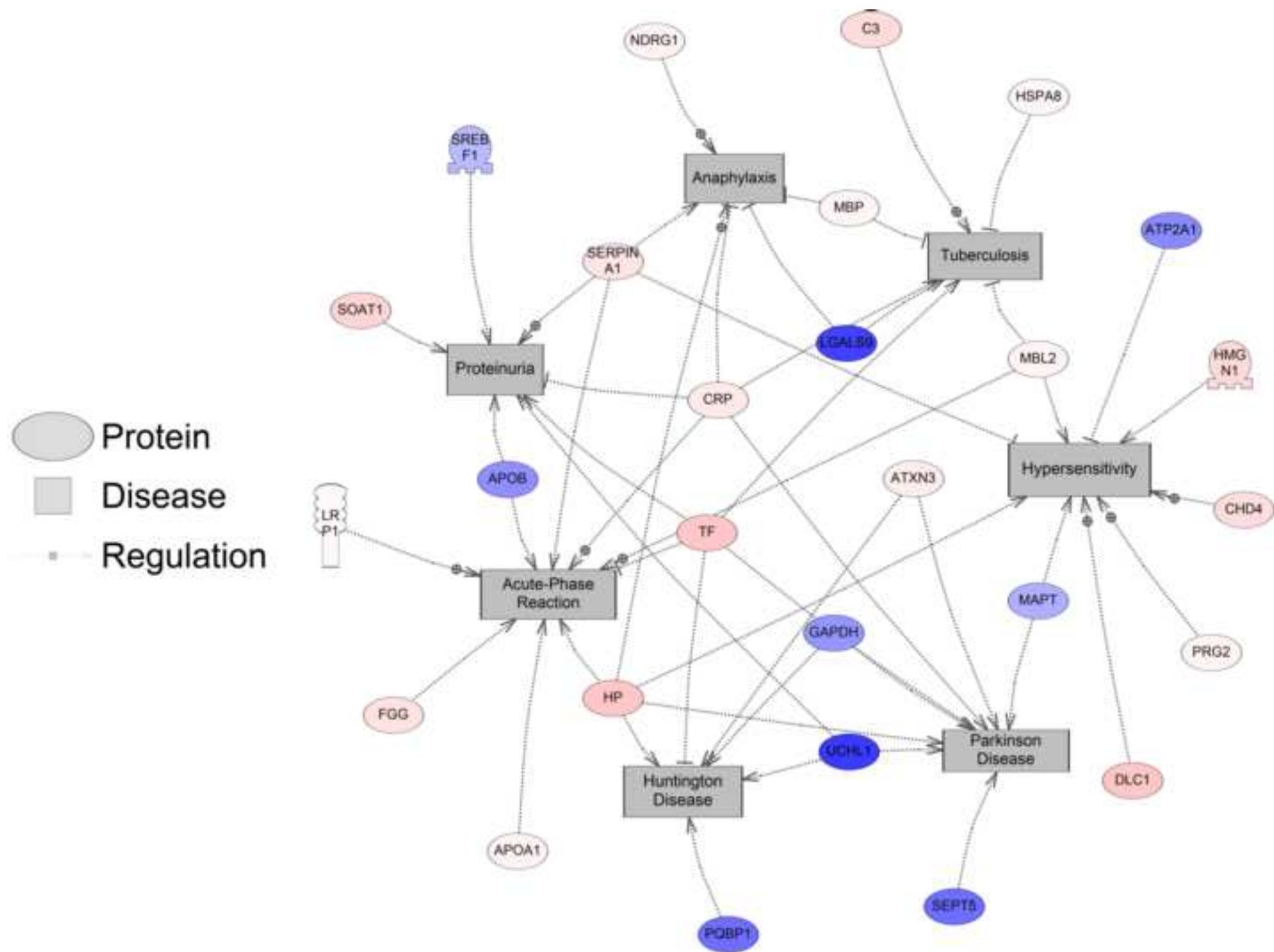


figure \*



## **Significance**

Dieldrin is a persistent organochlorine pesticide that has been associated with human neurodegenerative disease such as Parkinson's disease. Dieldrin is ranked 18<sup>th</sup> on the 2015 U.S. Agency for Toxic Substances and Disease Registry and continues to be a pesticide of concern for human health. Transcriptomics and quantitative proteomics (ITRAQ) were employed to characterize the molecular networks in the central nervous system that are altered with dietary exposure to dieldrin. We found that transcriptional and protein networks related to the immune system, mitochondria, and Parkinson's disease were preferentially affected by dieldrin. The study provides new insight into the mechanisms of dieldrin neurotoxicity that may explain, in part, the association between this pesticide and increased risks to neurodegeneration. These data contribute in a significant way to developing a molecular framework for pesticide induced neurotoxicity.

