Title: Proteomic Analysis of Zebrafish Brain Tissue following Exposure to the Pesticide Prochloraz

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Abstract

The hypothalamus-pituitary-gonadal (HPG) axis plays a central role in the maintenance of homeostasis and disruptions in its function can have important implications for reproduction and other critical biological processes. A number of compounds found in aquatic environments are known to affect the HPG axis. In the present study, we used 2-dimensional electrophoresis to investigate the proteome of female and male zebrafish brain after 96 h exposure to the fungicide prochloraz. Prochloraz has known effects on a number of key HPG molecules, including antagonism of Cyp17 and Cyp19 (aromatase). Twenty-eight proteins were shown to be differentially expressed in the brains of females and 22 in males. Proteins were identified using LC-MS/MS and identities were examined relative to brain function in the context of changing steroid hormone levels. There was little overlap between sexes in proteins exhibiting differential expression. Proteins with known roles in metabolism, learning, neuroprotection, and calcium regulation were determined to be differentially regulated. Relationships between identified proteins were also examined using Ingenuity Pathway Analysis, and females were shown to exhibit enrichment of several metabolic pathways. We used differentially expressed proteins to establish a putative classifier consisting of three proteins that was able to discriminate prochloraz-exposed from control females. Putatively impacted brain functions and specific protein changes that were observed have the potential to be generalized to other that similarly impact steroid hormone levels.

Keywords

Proteomics, brain, hypothalamus/pituitary/gonadal, prochloraz, zebrafish, gene expression

1. **Introduction**

There has been growing concern regarding unanticipated adverse effects resulting from exposure to pesticides, pharmaceuticals and personal care products. These chemicals are often active at relatively low concentrations in aquatic environments and can negatively impact a wide range of invertebrate and vertebrate species. Moreover, exposures often occur as part of complex mixtures (Gilliom 2007), which presents a problem for risk assessors who are tasked with identifying and characterizing complex ecological exposures. Methods are thus needed to reduce the complexity inherent in exposure assessment and characterization in aquatic environments. Potentially, this could be accomplished by grouping chemicals according to mechanism of action (MOA), effectively reducing the number of analytes from 1000s of chemicals to tens or hundreds of MOAs or pathways. In addition to the reduction in complexity, this provides a biological context for the exposure. Further, it allows an unbiased means to measure the total degree of exposure, thereby making it possible to link the occurrence of a chemical with apical endpoints, such as reproduction, in terms of understanding and predicting environmental impacts. A well-known example of this type of integration is the measurement of gene and/or protein expression of vitellogenin in fish as a means of quantifying the degree of estrogenic exposure.

In vertebrates, the hypothalamus represents a master regulator of homeostasis and is the critical nexus between the nervous and endocrine systems. The hypothalamus mediates responses to homeostatic imbalance mainly through regulation of the pituitary gland, which, in turn, produces hormones that are able to affect systemic change, for example within the gonad. The central role of the hypothalamic-pituitary-

gonadal (HPG) axis makes it particularly susceptible and sensitive to perturbation by a variety of environmental contaminants. Chemical disruption of the HPG axis will often result in modifications of circulating hormones, leading to an inability to mitigate environmental stress, as well as, direct impacts on reproduction and development, which has been demonstrated to produce population-level impact on fish (Kidd et al. 2007; Miller et al. 2007). Measurement of disruption of various components of the HPG axis following chemical exposure provides a convenient means to evaluate the shared MOA concept. For these reasons, a multi-laboratory collaborative project has focused on alterations of the HPG axis to identify relevant perturbations on multiple biological levels of organization using model chemicals known to impact key regulatory molecules within the HPG axis (Ankley et al. 2009a; Ankley et al. 2009b).

The current work focuses specifically on the impact of prochloraz (PCZ) on the brains of exposed zebrafish. PCZ is an imidazole fungicide that inhibits Cyp51 (lanosterol 14α-demethylase), an important enzyme in ergosterol synthesis from squalene, resulting in weakened fungal cell membranes (Henry and Sisler 1984). Prochloraz is considered an endocrine disrupting compound (EDC) and its impacts on the HPG axis have been relatively well studied. It has been shown to inhibit Cyp17, the enzyme responsible for conversion of progesterone to androstenedione (Blystone et al. 2007) and Cyp19 (aromatase), which converts testosterone to 17β-estradiol (E2) (Hinfray et al. 2006). It is also able to antagonize androgen receptor (AR) and estrogen receptor (ER) activity (Andersen et al. 2002). Together the inhibition of steroidogenic Cyp enzymes and antagonism of steroid hormone receptor activity results in altered levels of the circulating sex hormones testosterone (T) and 17β-estradiol (E2). PCZ-

dependent depression of plasma T and E2 levels has been previously observed in fish models (Ankley et al. 2005; Ankley et al. 2009b) and these alterations were linked to reduced reproductive success (Ankley et al. 2005; Zhang et al. 2008). Due to the observed effects on circulating hormone levels and the well defined role that these hormones play in the brain, it is possible that at least part of the endocrine disrupting effect of PCZ is due to interactions with the CNS of exposed organisms. However, the nature of these interactions or their underlying molecular mechanisms remains largely uncharacterized. Therefore, characterization of the interactions of PCZ with the CNS requires an experimental model that would integrate both direct and indirect systemic effects. In the present study, we employed the zebrafish (*Danio rerio*) as a model experimental system to characterize the interactions of PCZ with the CNS.

Following ligand binding, activated nuclear hormone receptors are translocated to the nucleus where they initiate gene transcription. Newly transcribed genes are subsequently translated into proteins, which play active roles in the biological response. Hence examination of gene or protein changes following exposure to compounds that lead to the disruption of hormone levels is a relevant approach to evaluating downstream effects. Furthermore, the identification of responsive genes and proteins may be valuable in interpreting the MOA of chemicals that disrupt the HPG axis. Two-dimensional electrophoresis (2-DE) has been shown to be a useful tool for the interrogation of the proteome of affected tissues in a non-biased manner (Shepard et al. 2000; Biales et al. 2010). The establishment of a protein expression signature for PCZ, as a model chemical for disruption of the HPG axis, may provide a practical tool for the identification of aquatic exposures from chemicals that affect circulating hormone levels.

In the present study, we used 2-DE to identify proteins differentially expressed in the brains of female and male zebrafish exposed to PCZ for 96 h. We examined the identity of these proteins relative to defined pathways and biological processes to characterize the modification of CNS function by PCZ. We also examined the ability of differentially expressed proteins to discriminate between exposed and control organisms, with hopes of providing a useful biomarker for disruption of HPG function that could be used in assessments of aquatic ecosystems.

2. Materials and Methods

2.1 Culturing and Exposures

Reproductively mature male and female zebrafish (5 months old, *ab* strain) used in this experiment were obtained from the on-site culture facility of the US EPA National Health and Environmental Effects Laboratory in Duluth, MN. Fish were cultured and exposed in UV sterilized, 0.1 μm filtered, Lake Superior (USA) water pumped from approximately 200 m offshore of the laboratory (Duluth, MN) at a depth of 20 m. All zebrafish exposures and laboratory procedures performed in this experiment were approved by the laboratory's Institutional Animal Care and Use Committee.

Three days prior to the initiation of dosing, zebrafish were randomly transferred to aquaria receiving control Lake Superior water and allowed to acclimate to the experimental conditions. Lake Superior water without (control) or with PCZ directly dissolved in water (500 μg/l, nominal; 99.5% purity; Sigma, St. Louis, MO) was delivered to exposure tanks, at a continuous flow rate of 45 ml/min for 48 hours, to allow the stabilization of prochloraz concentrations prior to the addition of test organisms (n=5 tanks per treatment). Following stabilization, exposures were initiated by transferring 10

male and 10 female zebrafish from each acclimation tank to each of five replicate exposure tanks (n=10 males and 10 females/tank: n = 50 male and female /treatment; see Supplementary Figure 1 for sampling schematic). In order to increase sampling efficiency, exposure tanks were divided into two compartments with a water permeable barrier, providing physical separation of males and females. Prochloraz concentrations were determined from 1 ml water aliquots collected from each of the exposure tanks at times 0, 24, 48, 72, and 96 h of the test using high-pressure liquid chromatography with diode-array detection at 220 nm as previously described (Ankley et al. 2005).

After 96 h of exposure, four male and four female zebrafish were sampled from each of the five exposure tanks (n = 20/sex) and euthanized with an overdose of tricaine methanesulfonate (Finquel MS-222; Argent Chemical Laboratories, Inc., Redmond, WA). Ninety-six hours was selected as the time point of interest in order to allow adequate time for hormone level and protein expression changes to occur. Whole brain (brain and pituitary) tissues were removed from fish using dissecting tools cleaned with RNAseZAP® (Ambion, Inc., Austin, TX) between each sample and transferred to microcentrifuge tubes (Ambion) containing 3.2 mm stainless steel beads (Biospec Products, Bartlesville, OK) and immediately snap-frozen in liquid nitrogen. All tissue samples were stored at -80 °C until analyzed.

2.2 Protein isolation

Brain samples used in 2-DE were randomly selected from the 20 per sex/treatment isolated (described above; Supplemental Figure 1), but with at least one sample per sex from each of the five replicate exposure tanks per treatment (n=6 per

sex/treatment). Remaining fish were used for other analyses. Proteins were isolated from male and female brain samples from control and treated zebrafish. Brain samples were individually homogenized in 300 µl of difference in-gel electrophoresis lysis buffer (DIGE LB; 30 mM Tris, 2 M thiourea, 7 M urea, 4% CHAPS. pH 8.80 with HCl) by shaking at 30 Hz for 5 min in a MM300 mixer mill (Retsch Laboratory Equipment, Newtown, PA). Homogenates were centrifuged for 5 min at 14,000 g, 4 °C, to remove any remaining debris and insoluble material. Supernatants were transferred to new 1.5 ml microcentrifuge tubes. Protein concentrations were determined using the EZQ™ Protein Quantification Kit (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's protocol. Fluorescent spots on membranes were visualized using a Typhoon 9410 variable mode imager (G.E. Healthcare, Piscataway, NJ) and quantified using ImageQuant TL software (G.E. Healthcare) and a purified ovalbumin standard curve. Following quantitation, aliquots of each brain homogenate (125 µg) were purified in duplicate using the 2-D Clean-up Kit (G.E. Healthcare) following the manufacturer's protocol. Protein samples were resuspended in 20 µl of DIGE LB and incubated overnight at 4 °C. Duplicate samples were subsequently pooled, re-quantified, and stored at -80 °C until further use.

2.3 Protein Labeling and Electrophoresis

All protein labeling and electrophoresis procedures followed manufacturer's protocols and were performed as previously described (Biales et al. 2010). Briefly, aliquots (35 µg) of all brain samples (male and female, treated and control) were combined to generate a protein standard pool. This pool and the individual male and

female brain samples were labeled with fluorescent cyanine dyes (CyDye™ DIGE Fluors; G.E. Healthcare), using the Minimal Labeling kit (G.E. Healthcare). Six male and female experimental samples (50 µg) from each treatment group were labeled with 300 pmoles of either Cy™3 or Cy5, while the standard pool was labeled with Cy2 (same protein mass to dye ratio). Half (n=3) of the brain samples from each unique experimental group were labeled with Cy3 and half with Cy5, to control for any potential dye bias. Within each sex, Cy3- and Cy5-labeled brain samples were combined (randomly) with a 50 µg aliquot of the Cy2-labeled standard pool, loaded onto 24 cm immobilized pH gradient (IPG) strips (pH 4-7, G.E. Healthcare), and subjected to first dimension iso-electric focusing (IEF) overnight using the ETTAN™ IPGphor™ II system (G.E. Healthcare). Upon completion of the IEF, the IPG strips were stored individually in sealed plastic tubes at -80 °C. Prior to the second dimension electrophoresis, IPG strips were thawed at room temperature, equilibrated twice for 10 min with constant mixing (60 rpm), first with 0.5% dithiothreital (DTT) and subsequently with 4.5% iodoacetamide (IAA). The IEF strips were immediately overlaid onto precast 24 cm, 1 mm thick, 12% polyacrylamide Laemmli gels (Jule Inc., Milford, CT), sealed with 0.8% agarose, and electrophoresed at 2 W per gel, at 10 °C, for 16.5 h on an ETTAN™ DALT*twelve* Large Vertical System (G.E. Healthcare).

2.4 Image Acquisition and Processing

Following electrophoresis, gels plates were washed with 70% ethanol followed by distilled water. A total of 12 gels (six containing male samples and six containing female samples) were scanned using a Typhoon 9410 Variable Mode Imager at 1000

μm in each of the three channels (for the three CyDyes). Laser intensities were adjusted per channel per gel and a subsequent final scan was performed in each of the three channels at 100 μm. Gel images were cropped using ImageQuant TL v2005 and imported into DeCyder v 6.5 (G.E. Healthcare) for matching and all subsequent processing and analyses unless otherwise noted. Images were processed using the Difference in Gel Analysis module (DIA) of the DeCyder software. Processed DIA images were then imported into the Biological Variation Analysis (BVA) module for inter-gel matching. Separately, the Cy2 standard images from each sex were examined and the images that displayed the fewest experimental artifacts and had the highest resolution over the entire gel were selected as the master image for each sex. All other gels within a sex were subsequently matched to these gels. Different master gels were selected in the male and female datasets to increase the likelihood of finding differentially expressed proteins independent of technical artifacts. However, using different master gels between genders had the consequence of making direct quantitative comparisons of protein spots between genders impossible. Before automated gel matching, the Cy2 standard images from all gels were extensively landmarked, targeting edges, to minimize mismatches. Match fidelity was visually confirmed for a subset of spots from areas of high fluorescence and from edges. In cases where automated matching was of low quality, gels were unmatched, additional landmarks added, and then re-matched using the automated matching feature.

2.5 Statistical analysis:

Following matching, spot data were imported from the BVA into the Extended Data Analysis (EDA) module of the DeCyder software for data analysis. All data analyses were performed on log standardized abundance values. Data were filtered so that only those spots that were present in 75% of the spot maps (biological replicates) between the control and PCZ treated individuals of females and males, respectively, were included in the analyses. All statistical analyses were done using the EDA module. A t test ($p \leq 0.05$) was conducted on the log standardized abundance values to identify proteins that were differentially expressed between controls and treated individuals for each sex independently. The fidelity of matching for all differentially expressed proteins was visually confirmed in the BVA module.

2.6 Protein Identification

Preparative (spot picking) gels were prepared essentially the same as detailed in Biales et al. (2010). Two preparative gels each were run for males and females. For preparative gels, 525-600 μg of protein was loaded on to each IPG strip. Proteins used for preparative gels were from pools of four individuals each. Fish used for preparative gels were obtained from the same stock as those used in 2-DE, however, at a later date. Following the second dimension, these gels were post-stained with Deep Purple Total Protein Stain (GE Healthcare) following the manufacturer's recommended protocol. Gel plates were cleaned and scanned as described above. Following scanning, gel images were processed using the DIA module and imported into the existing BVA workspace. Proteins to be excised were manually matched between the preparative gel and the respective master gel. Spots were excised using the Ettan Spot

Picker (GE Healthcare), placed into a 96-well plate, sealed and stored at 4 °C. Accuracy of spot picking was evaluated by rescanning the gels. Gel plugs were shipped overnight on dry ice to the University of Florida, Interdisciplinary Center for Biotechnology Research Proteomics Division for protein identification. Proteins were purified and digested and LC-MS/MS analyses were performed as previously described (Biales et al. 2010).

2.7 Database Searching:

Tandem mass spectra were extracted by ABI Analyst version 1.1 (Applied Biosystems). Charge state deconvolution and deisotoping were performed in the background of the Analyst software. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version Mascot). X! Tandem (www.thegpm.org; version 2007.01.01.1) was only used to validate samples. Mascot was set up to search the NCBInr database (NCBInr_20100312.fasta selected for Danio rerio, 44543 entries) assuming the digestion enzyme trypsin. Iodoacetamide derivative of cysteine was specified in Mascot and X! Tandem as a fixed modification. S-carbamoylmethylcysteine cyclization (N-terminus), deamidation of asparagine and oxidation of methionine were specified in Mascot as variable modifications. S-carbamoylmethylcysteine cyclization (N-terminus), deamidation of glutamine and oxidation of methionine were specified in X!Tandem as variable modifications.

2.8 Criteria for protein identification:

Scaffold (version Scaffold_2_04_00, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide

identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm (Keller et al. 2002). Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least two unique peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al. 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins identified as keratin were considered artifacts from contamination. When database searches matched multiple proteins, the protein match that had the largest number of unique peptides and the greatest percentage coverage was considered correct. For those matches where replicate spots were sequenced from different preparative gels, only those identities that were in common between the replicates were considered and then these were selected based on the criteria above. If proteins did not match between preparative gels, then "replicates did not match" was recorded. In those cases where no one protein match could be selected, all top matches were reported. If multiple accession numbers for the same identification were listed, the one with the largest degree of annotation was used.

2.9 Discriminant analysis

To determine if proteins would be useful as a group for differentiating exposed and naïve zebrafish, a principal component analysis (PCA) was conducted. Discriminant analysis was carried out identically for females and males. Protein spots that were identified as differentially expressed in a Student's t test were filtered based on p value and only those significant at $p \leq 0.01$ were included in the discriminant analysis. Protein sets were used as loadings for PCA of female and male datasets.

Additionally, protein sets were evaluated in pairwise hierarchical clustering based on the Euclidean distances of their respective expression values using the default settings in the EDA module. To further evaluate the proteins used in the PCA as potential biomarkers for PCZ exposure, the marker selection and classifier creation functions of the EDA were used. Both partial least squares (PLS) and forward selection (FS) were used for the search methods, and K-Nearest Neighbor (KNN) or Regularized discriminant analysis (RDA) were selected as the evaluation method. Several sets of proteins were created based on the results of the marker selection. These different sets of proteins were then evaluated for their ability to discriminate PCZ-exposed from control individuals using leave-one-out-cross-validation (LOOCV) and KNN or RDA as the classification method. LOOCV trains the model based on k-1 folds and uses one fold for testing. This is done iteratively so that each individual is used as a test to evaluate the accuracy of the model. LOOCV was used because the dataset (biological replicate number) was too small to create an independent test set.

2.10 Network Pathway Analysis

We used Ingenuity Pathway Analysis (IPA: version 9.0, Ingenuity Systems, www.ingenuity.com) to determine functional relationships among identified proteins, as well as to determine significant enrichment of canonical pathways and known biological functions. Female and male datasets were analyzed independently. Identifiers for differentially expressed proteins were uploaded into the IPA software and mapped to corresponding molecules in the Ingenuity Knowledge Base. All entries in the knowledgebase are from human, mouse or rat. Only unambiguously identified proteins were included in the IPA. Duplicate entries were only counted once. Analyses included

only nervous system tissues and CNS cell lines. The significance level for enrichment of canonical pathways and biological functions was determined using the Benjamini-Hochberg multiple test corrected value. Both uncorrected p values and the B-H value are reported. Though female and male analyses were conducted independently their respective identified networks were overlaid on a single figure to illustrate connections.

3. Results

3.1 Differential expression

Female spot data were exported from the BVA module into the EDA module and subsequently filtered so that only those spots that were present in at least 75% of the gels were included in further analysis, resulting in 1404 protein spots. A Student's t test was then conducted between female PCZ-exposed and control brains and 28 spots were shown to be significantly different ($p < 0.05$) and visually confirmed (Figure 1a). The magnitude of difference in brain protein abundance between PCZ-exposed and control fish ranged from 2.02 fold up- to 2.23 fold down. Nineteen spots were observed to be up-regulated, whereas only 9 were down-regulated (Table 1). Male brains were analyzed independently, using the same approach as for females. Upon import into EDA there were 2007 protein spots, of which, 1290 remained after filtering. Twenty-two spots were determined to be differentially expressed between the control and PCZ-treated males (Figure 1b). The magnitude of change ranged from 1.69 fold up- to 1.89 fold down, with 15 proteins up- and 10 proteins down-regulated. As different master gels were used between the female and male analyses, comparisons between the two datasets were qualitative and limited to only those spots that were positively identified using LC-MS/MS.

3.2 Spot identification

In all, 64 spots from the four preparative gels were excised and sequenced. Of the 32 spots for each gender, there were 21 unique and 11 duplicate spots for females and 20 unique and 12 duplicate spots for males. Sequencing failed for 3 replicates (two female and one male) and there was disagreement between a single replicate sequencing attempt in each dataset. The identities of these mismatch spots were considered unknown and are reported as "replicates did not match" in Table 1. The overall strong agreement in identification between spots picked from replicate preparative gels indicated that spot calling and picking were done accurately and consistently.

While seven of the 28 spots identified as differentially expressed in the female brains were not able to be picked and sequenced, at least one replicate of the remaining 21 unique spots yielded acceptable sequence matches. Two spots from the female dataset matched with more than one protein in the database, spots 672 and 985, and both potential identities are listed in Table 1. One spot, 495, aligned with two proteins, apolipoprotein B and an internexin neuronal intermediate filament protein. The MW of apolipoprotein B was predicted to be approximately 500 KDa, which was considerably larger than what was resolved on the gel, where the maximum resolution was expected to be around 100 KDa. Therefore, apolipoprotein B was not considered as a possible match and internexin intermediate filament protein was assigned as the identity of spot 495. Of the 22 differentially expressed proteins in the male brains, 20 were picked and sequenced and only spot 674 failed to yield an acceptable protein

match. Two protein spots, 702 and 652, were both identified as Dypsl3; however, spot 652 was identified as Dypsl3 in two independent preparative gels while spot 702 was only identified from a single gel. Spot 702 was also identified as another member of this protein family, Dypsl 5, with similar scores (Dypsl3 vs Dypsl5; percent coverage, 25.7 vs 17.6; number of unique peptides, 12 vs. 10). For these reasons, we considered spot 652 positively identified as Dypsl3 and spot 702 as Dypsl5. One protein was found to be differentially expressed in both the female and male datasets, a hypothetical protein (GI:189529246). Using the Conserved Domain Database search (http://www.ncbi.nlm.nih.gov/cdd), this protein was found to contain a conserved apolipoprotein domain.

3.3 Network Pathway Analysis.

For females, of 17 proteins uploaded into the IPA, 15 were matched to a corresponding protein in the Ingenuity Knowledgebase. All were identified as network eligible proteins and 14 were eligible for functions or pathways.

Three networks were initially identified; however, one (organismal functions, cell death and neurological disease: score = 9) had a significantly higher score (9 vs. 2) than the remaining two networks, thus discussion was limited to this network (Figure 2). The female dataset was found to be enriched for proteins from several canonical pathways, namely glycolysis/gluconeogenesis (p value = 8.94^{-5}, B-H =0 .003), fructose and mannose metabolism (p value = 0.001,B-H = 0.02) and pyruvate metabolism (p value = 0.002, B-H = 0.03)

Differentially expressed proteins identified in male FHM were processed identically as females. For males, 18 proteins were entered and 13 were mapped. The five unmapped proteins were: a predicted protein, lipocalin-type prostaglandin D synthase-like protein, Dypsl5, EPD and DDAH1. A single enriched network was identified, namely, genetic disorder, neurological disease, skeletal and muscular disorders (Score=11) (Figure 2). As was observed in females, Gycolysis/Gluconeogenesis was the most enriched canonical pathway, though upon multiple test correction, it was not significant (p value = 0.004, B-H = 0.12). Overall, relationships among proteins and to canonical pathways appeared to be reduced in the male fish relative to the females; however, it is unclear what effect the inability to map proteins (5/18 proteins) had on interpretation of the male IPA.

3.4 Discriminant analysis

A PCA based on the expression of 11 protein spots was conducted for the females (Supplemental Figure 2a). There was good separation between the PCZ-exposed and control zebrafish, with 70.1% of the variability explained by the first axis and an additional 18.8% explained by the second. This degree of separation between treatment groups was supported by the hierarchical clustering, where all of the PCZ-exposed individuals clustered together on a separate node than the control individuals (Figure 3a). Taken together, this suggests that differentially expressed proteins may be useful for discriminating exposed from naïve organisms.

There was a similar degree of separation seen in the PCA of the males, where 86.8% of the variability was explained by the first axis and 9.5% by the second

(Supplemental Figure 2b). The total cumulative variability reached 99% by the third axis. Hierarchical clustering of the males did not appear as distinct as what was observed for the females (Figure 3b). Four of the PCZ-exposed males clustered on a node separate from the controls; however, two others were on a node by themselves and clustered more closely to the controls. There were no obvious technical reasons why these two individuals did not group with the remaining exposed males. Based on the heat maps, it appears that protein spot 271 accounted for the apparent misclassification of the two individuals; however, it should be noted that this analysis was based on only four protein spots. Therefore, variability surrounding any one protein will likely have a comparatively large affect.

Proteins used for loadings in the PCA were then evaluated using the marker selection feature of the EDA. The results of the marker selection for the female dataset suggested just three proteins were sufficient to discriminate between PCZ-exposed and control individuals (Supplementary Figure 3a). Four sets of proteins were evaluated on their discriminative ability, containing one, three, four and eleven proteins respectively. A minimum of three proteins were shown to be needed for 100% accuracy in the LOOCV evaluation using either the KNN and RDA classification method (Table 2). These proteins were 985, 2033 and 2092, unfortunately only spot 2092, DJ-1, was positively identified in LC-MS/MS (Table 1). Male proteins were evaluated similarly to the females. The marker selection feature suggested that all four proteins would be needed for 95% accuracy (Supplementary Figure 3b); however, when evaluated in the LOOCV using either KNN or RDA as the classification method, the classifier was found to be less accurate than the female classifier (90% ± 22.4 vs 100% ± 0).

4. Discussion

We have demonstrated that PCZ is able to induce changes in the proteome of the zebrafish brain. Up- and down-regulation of proteins was observed in both female and male brains following a 96 h exposure (Table 1). The majority of proteins were up-regulated in females (19 out of 28), whereas, males displayed a more equal distribution of up- and down-regulation.

Overall the magnitude of change of protein expression in both male and female PCZ treated fish relative to untreated fish was relatively small. The range in fold change was shown to be slightly greater in the female than the male (Table 1). The current study examined protein expression in whole brain and the magnitude expression observed here is consistent with other studies also using whole brain (Biales et al. 2010; Szego et al. 2010). The brain is highly complex, with functions being relatively regionalized. The magnitude of expression observed in the current study may result from an averaging effect across different brain regions. Supporting this is the observation that brain tissue exhibits regional variation in gene and protein expression concomitant with modulation of circulating hormone levels (Mitev et al. 2003; Yamada et al. 2009), which aligns with the main MOA of PCZ.

4.1 Sex-specific differences in expression

There was little overlap between sexes in the specific proteins shown to be differentially regulated, as only one protein, a hypothetical protein (GI:189529246), was shown to be in common. However, network analysis identified indirect relationships between the female and male datasets, primarily centered on the proteins discs, large

homologue 4 (DLG4) and huntingtin (HTT) (Figure 2). This may indicate that similar cellular functions were altered in both sexes. Differences in proteomic profiles, both the specific proteins altered and the direction of regulation, may indicate a sex-specific response to PCZ. Sex-specific differences in gene expression in fish resulting from chemical exposure have been observed previously (Gomiero et al. 2006; van der Ven et al. 2006). Sex by itself was found to be a major factor for differential gene profiles in zebrafish exposed to HPG-active chemicals with different MOA (Wang et al. 2008). Further, the effects of PCZ on circulating T and E2 levels in fish differ in a sex-specific manner (Ankley et al. 2005), which may underlie the observed gender differences in protein profiles. Sex specific differences in estrogen receptor expression, both in respect to density and regional expression, have been observed in rats in response to estrogenic exposure (Yamada et al. 2009) and circulating hormone levels are known to have major and gender specific roles in a number of aspects of brain function (reviewed in (Gillies and McArthur 2010))

4.2 Metabolic response

Adaptation to environmental stress comes at a metabolic cost, as it has been linked to glycogen depletion in brains of pesticide exposed fish (Sarin and Gill 1999). Given that cellular energy resources are limited, a balance must be reached in regard to energy usage for mitigation of the stress event versus maintenance and growth. In the present study, we have identified a number of differentially expressed proteins known to be associated with the glycolytic pathway (ALDH2, ALDH7, HEX1, PKM2a and PGM1) and ATP production (PYCb, ETFa, ATP5a). Statistically significant enrichment in the glycolytic/glyconeogenic pathway was also observed in IPA analysis for both female

and male (before B-H correction) datasets. Differential regulation of these proteins may suggest that brain tissue in exposed organisms is compensating for the increased energy requirement resulting from PCZ toxicity. Prochloraz exposure has also been shown to increase energetically costly activities such as the number of burst swimming bouts and antagonistic acts (Saglio et al. 2001; Saglio et al. 2003). Moreover, these behavioral changes coincided with reduced feeding attempts (Saglio et al. 2001). Taken together, mitigation of toxicity and increased activity, coupled with decreased feeding, would be expected to impose a considerable energy burden on PCZ-exposed organisms and reduce investment in growth and reproduction.

4.3 Heat Shock Proteins

Differential expression of stress response proteins was observed in the brains of PCZ-exposed male and female zebrafish. Stress response proteins are a group of highly conserved cellular proteins that are involved in tolerance to a number of stressors (Iwama et al. 1998; Feder and Hofmann 1999), including pesticides known to impact the nervous system (Eder et al. 2007; Eder et al. 2009). Heat shock proteins (HSPs) are considered molecular chaperones, and thus play a critical role in the folding, stabilization and refolding of cellular proteins, as well as targeting proteins to specific cellular compartments. Moreover, HSPs have been shown to be expressed in response to a number of stressful conditions. In the current study, HSP90 was shown to decrease in abundance in response to PCZ exposure in the male. This protein plays a central role in the stress response and is pivotal in a number of cellular signaling pathways. HSP90 has been shown to interact with and mediate the signaling of a number of nuclear receptors including the estrogen (Gougelet et al. 2005), androgen

(Pratt et al. 2004) and progesterone receptors (Felts et al. 2007). As many steroid hormone nuclear receptors are known to be autoregulated (Cardone et al. 1998; Matthews and Gustafsson 2003), their gene and protein expression would likely be altered in response to chemicals, such as PCZ, that target steroidogenic enzymes. Differential expression of a number of hormone receptors, including estrogen and androgen receptors, has been observed in medaka exposed to PCZ and ketaconazole (Zhang et al. 2008). HSP90 expression has also been shown to be up-regulated following estrogen exposure (Stein et al. 2001). Thus a reduction in HSP90 abundance may result from a PCZ-dependent down-regulation of either hormone receptors or the hormones themselves. Interestingly, Cdc37 was shown to decrease in abundance in PCZ exposed females. This protein is a known binding partner of HSP90 and targets the HSP90 complex to specific cellular kinases (Stepanova et al. 1996). The observation that Cdc37 displayed decreased abundance in the female and HSP90 in the male, may suggest that the HSP90 complex is being targeting to different nuclear receptors in females and males and may underlie the mechanism for differential responses of males and females to PCZ.

4.4 Neuroprotection

Increased circulating sex hormone levels are associated with neuroprotection (Zhang et al. 2004; Soustiel et al. 2005; Zhao and Brinton 2007), whereas reductions in estrogen levels have been linked to increased apoptosis in brain tissue (Hill et al. 2009). We have identified several proteins in both the female and male datasets that are known to regulate apoptosis in the brain. In the female dataset, DJ-1 (Parkinson disease 7) was observed to be down-regulated (Table 1). This protein acts as an anti-

apoptotic protein (Bretaud et al. 2007), thus its decreased abundance may suggest increased levels of apoptosis in PCZ-exposed females. The top network identified for the female dataset, 'organismal functions, cell death, neurological disease', further suggests a role for a PCZ effect on apoptosis in brain tissues. The pattern of differential expression of apoptotic-related proteins in PCZ exposed males is less clear, since up-regulation of both pro- (programmed cell death 6 interacting protein; PCD6IP) and anti-apoptotic (HSPA9) proteins was observed. Sex-specific differences in apoptotic related proteins may suggest that males are less sensitive to this aspect of PCZ toxicity or that a neuroprotective phenotype is more dependent on estrogens than androgens, which is consistent with the observation that PCZ exposed male fathead minnows demonstrated no reduction in circulating levels of E2, whereas females did (Ankley et al. 2005).

4.5 Cognitive effects

In addition to its direct role in neuroprotection, estrogen is also associated with increased learning, sensory input and processing and memory consolidation. Positive effects of estrogen on aspects of cognitive behavior may be related to observed alterations in synaptic structure and function (Smith et al. 2010). In IPA, DLG4 was identified in networks from both female and male datasets (Figure 2). This protein is associated with enhancement of pre and post-synaptic signaling and has been shown to be differentially-regulated by estrogen treatment in mice (Li et al. 2004). DLG4 has been shown to form multimeric complexes with a number of proteins identified here as displaying altered abundance in response to PCZ, namely ATP5b, PKM2, GLUL and DJ1.

Changes in abundance of several other proteins associated with synaptic function in the CNS were also observed. Dypsl3 and Dypsl5 are members of the collapsin response mediator protein family, which are involved in axonal guidance during development and are expressed in regions of the brain associated with neuronal plasticity and neurogenesis in the adult (Charrier et al. 2003; Veyrac et al. 2005; Cnops et al. 2006). SNAP25 and ependymin both decreased in abundance in response to PCZ exposure. SNAP25 is a member of the Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex, which plays a role in neuronal vesicular trafficking (Tafoya et al. 2006) and SNAP25 expression has been shown to vary with estrogen levels (Pechenino and Frick 2009) or exposure to chemicals known to alter circulating estrogen levels (Zhang et al. 2009). Ependymins are extracellular glycoproteins identified in the teleost brain and are associated with learning and memory consolidation, as well as neuronal regeneration (Shashoua 1991). They have been shown to be up-regulated following exposure to EE2 in fathead minnows (Martyniuk et al. 2010), which is consistent with the decrease in abundance observed in the current study resulting from the presumptive decrease in estrogen levels.

4.6 Calcium regulation

Ca^{2+} plays a central role in neuronal function and maintenance and its dysregulation is associated with a number of neurological disorders (Thibault et al. 2007; Marambaud et al. 2009). Increased Ca^{2+} levels following ER activation were shown to be required for activation of neuroprotective pathways in primary neuronal cultures (Zhao and Brinton 2007). Not surprisingly, we have identified a number of proteins associated with Ca^{2+} in female and male exposed zebrafish. The Ca^{2+} sensing

proteins NECAB2 and PPID (female) and grancalcin and SNAP25 (male) were found to be differentially regulated in the present study (Table 1). Other studies examining the effects of chemicals targeting steroidogenic enzymes have also observed the differential expression of Ca^{2+} sensing proteins in the brain (Zhang et al. 2009). While it remains unclear what the exact role of Ca^{2+} fluctuation may be in PCZ toxicity, differential regulation of proteins associated with Ca^{2+} signaling in the brain may be useful in the construction of biomarkers for HPG axis disruption.

4.7 Biomarker development

The HPG axis represents a major nexus between systems of the body, and disruptions of HPG axis functions have been linked to reproductive effects in multiple vertebrate classes, such as mice, medaka and fathead minnows (Ankley et al. 2005; Vinggaard et al. 2005; Laier et al. 2006; Zhang et al. 2008). Reproductive or homeostatic impairment in natural populations of aquatic organisms such as fish could have important implications for the integrity of ecosystems. The development of biomarkers targeting specific molecular regulators within the HPG axis would be useful for relating the degree of exposure of chemical contaminants to apical endpoints (Ankley et al. 2009a). This information could then be used to generate predictive models to characterize the potential threat to ecological integrity. We have identified a putative classifier consisting of three proteins that was able to successfully discriminate PCZ-exposed female zebrafish from controls (Table 2). Though this classifier was shown to be successful here, further validation using an independent test set must be conducted to determine its true discriminative ability. The cellular processes that were shown to be affected by PCZ exposure in the present study have known linkages to

steroid hormones. This, in conjunction with the general overlap between the proteins observed in the present study and those seen in other studies targeting HPG axis disruption, suggests that they may be generalized to related compounds or classes of compounds that influence hormone levels; however, this claim has yet to be empirically tested. It also should be noted that PCZ has a number of possible alternative MOA outside of Cyp inhibition and antagonism of steroid hormone receptors. It is also been shown to activate the aryl hydrocarbon receptor (Babin et al. 2005) and reduce circulating levels of thyroxine and thyroid stimulating hormone (Andersen et al. 2002). Therefore, comparisons to other compounds with clearly defined MOAs within the HPG axis must be carried out to more accurately estimate the degree to which the identified biomarkers, in terms of affected brain processes, as well as, specific protein changes, can be generalized.

5. Conclusions:

We have shown that male and female zebrafish respond to the fungicide PCZ through alterations in their brain protein complement. The identity of these proteins provides information regarding the MOA of this fungicide; however, protein identification should be viewed as putative until confirmed using alternative experimental techniques. Prochloraz caused alterations in the abundance of several heat shock proteins as well as energy associated proteins. These may be indicative of a general stress response and not specific to PCZ; however, proteins known to be associated with neuroplasticity and neuroprotection were also differentially expressed. Changes in the abundance of these proteins have been previously linked to alterations of circulating hormone levels and may have important implications in the ability of exposed organisms to maintain

homeostatis. We have evaluated a subset of the differentially expressed proteins for their ability to discriminate exposed from control organisms in hopes of generating a usable classifier for HPG axis disruption. Further work will examine the ability of the classifier and the affected brain functions to be generalized for other compounds targeting specific foci within the HPG axis, especially those affecting steroid synthesis. The development of a usable biomarker for HPG axis disruption may have important practical implications for the accurate assessment of risks posed by these and related chemicals.

Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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Figure Legends

Figure 1: Typical gel images for male and female zebrafish exposed to prochloraz. Gels for (a) female and (b) male Gels were poststained with Deep Purple. Differentially expressed spots are numbered. Spot numbering is independent for male and female gels.

Figure 2: Ingenuity Pathway Analysis for female and male datasets. Female and male protein sets were analyzed independently using IPA. Top networks were identified and were overlaid to demonstrate points of connection between sexes.

Figure 3: Pairwise hierarchical clustering based on Euclidean distance for protein expression values of male and female zebrafish exposed to prochloraz. Proteins identified as differentially expressed in a Student's t test ($p \leq 0.01$) were used for a hierarchical clustering of (a) females and (b) males. Protein spot numbers are located on the y axis and individuals are on the x axis.

Table 1: Differentially expressed proteins for female and male zebrafish exposed to PCZ.

Female proteins

Spot ID	*Fold change*	*p value*	*Protein name*	*ACC*	*Theoretical MW/pI*	*Observed MW/pI*	*Unique peptides*	*Sequence coverage*
2033	2.02	1.07E-04	PREDICTED: hypothetical protein [Danio rerio]	189529246	14.0/6.2	12.3/5.8	4	20.2%
2021	1.83	1.06E-03	Triosephosphate isomerase 1b	47271422	26.8/6.9	12.4/5.9	14	61.3%
1879	1.75	0.0382	Nonspecific cytotoxic cell receptor protein 1 (Nccrp1)	77748006	27.2/5.6	16.1/5.6	3	11.4%
119	1.59	0.0371	-			101/6.4		
495	1.48	0.0222	PREDICTED: similar to internexin neuronal intermediate filament protein, alpha [Danio rerio]	189522988	64.9/4.8	75.9/5.3	10	21.5%
1116	1.4	0.0485	ATP synthase, H+ transporting, beta polypeptide ATP5b	159155102	55.1/5.14	44.2/5.3	15	36.2%
564	1.39	0.0179	Complement component C3-1	82524272	18.5/6.3	71.8/6.2	13	7.9%
603	1.37	0.0165	**Replicates did not match**			69.0/6.2		
14	1.29	8.74E-03	Pyruvate carboxylase (PYCb)	18858695	129.9/6.44	107/6.5	2	2.0%
31	1.29	1.78E-03	-			107/5.5		
259	1.28	0.0476	Fibrinogen, gamma polypeptide	47085769	48.8/5.1	90.4/4.9	2	3.9%
589	1.28	7.22E-03	Complement component (C3-1)	82524272	18.5/6.3	70.2/6.2	12	7.0%
390	1.24	0.0357	-			81.9/6.0		
249	1.23	4.10E-04	Hexokinase 1 (HEX1)	45501264	102.8/6.3	91.7/6.2	18	18.5%
672	1.21	0.0454	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	205831554	72.7/6.1	65.6/5.8	28	43.7%
			Transferrin	47264590	73.5/6.8		21	32.0%
2067	1.18	2.45E-03	-			11.6/57		
1944	1.14	0.0387	-			14.4/5.4		
1512	1.13	0.0285	Novel protein similar to vertebrate EF hand calcium binding protein 2 (NECAB2)	148725626	47.9/5.2	28.7/5.0	18	33.6%
1346	1.09	0.0366	Peptidylprolyl isomerase D (cyclophilin D)	50344784	41.3/5.8	34.7/6.0	12	22.6%
1179	-1.05	0.0331	Cell division cycle 37 homolog (CDC37)	41054768	43.4/5.1	41.9/5.2	9	21.0%
1761	-1.08	0.0468	Electron-transfer-flavoprotein, alpha polypeptide (ETFa)	38707985	35.1/6.9	20.2/5.9	13	41.4%
2085	-1.08	0.0184	Cytidine monophosphate kinase 1 (CMPK1)	150383502	22.4/5.5	11.1/5.6	10	57.7%
652	-1.1	4.27E-03	Transferrin	47264590	73.5/6.8	66.4/5.2	22	31.9%
1015	-1.11	0.0384	Aldehyde dehydrogenase 2 precursor (ALDH2)	20339358	56.6/6.1	48.3/5.7	17	28.1%
609	-1.25	0.0422	-			68.7/5.1		
1223	-1.37	3.27E-03	-			39.4/5.2		
985	-1.45	8.91E-03	Aldehyde dehydrogenase 7 family, member A1 (ALDH7a1)	27882244	55.6/5.9	50.0/6.2	13	28.6%
			ATP synthase subunit alpha, mitochondrial (ATP5a)	116325975	59.7/9.1		15	26.7%
2092	-2.23	2.24E-03	Parkinson disease protein 7 homolog (DJ-1)	54400374	19.8/5.8	10.8/5.9	8	42.9%

Male proteins

1453	1.69	0.013	Pyrophosphatase (inorganic) 1	220678153	32.7/5.2	33.6/5.2	11	40.9%
1496	1.59	0.015	**Replicates did not match**			31.8/5.2		
1782	1.44	0.0354	PREDICTED: hypothetical protein [Danio rerio]	189529246	14.0/6.2	18.9/8.5	11	53.8%
1806	1.34	0.0142	Grancalcin, EF-hand calcium binding protein	53933226	22.9/5.2	17.6/5.1	3	9.8%
773	1.29	0.0442	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 19 (DBP5 homolog, yeast)	27881976	54.7/5.6	60.8/5.6	4	6.8%
210	1.26	0.0291	Pyruvate kinase isozyme M1/M2 (Pkm2a)	45501385	58.1/6.4	89.3/6.1	2	3.4%
1878	1.24	0.018	Lipocalin-type prostaglandin D synthase-like protein	19911829	20.9/5.2	20.9/5.2	8	38.6%
1417	1.23	0.0206	-					
766	1.21	0.0413	Phosphoglucomutase 1(PGM1)	41056111	61.1/5.7	61.1/5.7	17	36.0%
578	1.17	0.0438	Heat shock protein 9 (HSPa9)	28278640	73.9/7.1	73.9/7.1	10	16.6%
702	1.15	0.0166	Dihydropyrimidinase-related protein 5 (Dypsl5)	66392186	61.3/6.4	61.3/6.4	10	17.6%
318	1.14	4.58E-03	Programmed cell death 6 interacting protein (PDCD6IP)	205277327	95.8/5.8	95.8/5.8	25	30.7%
1899	1.13	0.0345	Synuclein, gamma b	66472444	11.4/4.7	11.4/4.7	5	30.7%
1489	-1.09	0.024	Ependymin (EPD)	148724888	24.5/5.4	24.5/5.4	8	31.3%
1139	-1.15	0.0135	NSFL1 (p97) cofactor (p47)	55925383	40.7/5.0	45.8/5.0	12	33.1%
1129	-1.18	0.02	Glutamine synthetase b (GLUL)	225579106	41.9/6.0	41.9/6.0	16	34.4%
1724	-1.2	0.029	Synaptosomal-associated protein 25-A (SNAP-25A)	82196658	22.9/4.6	22.9/4.6	19	76.5%
652	-1.28	2.88E-03	Dihydropyrimidinase-related protein 3 (Dypsl3)	66472750	61.5/6.0	61.5/6.0	10	20.3%
271	-1.29	9.20E-03	Heat shock protein 90kDa beta, member 1 (HSP90)	38016165	91.3/4.8	91.3/4.8	23	27.6%
674	-1.36	0.0245	-					
1774	-1.43	0.0156	-					
1535	-1.86	0.0061	Dimethylarginine dimethylaminohydrolase 1(DDAH1)	47086113	28.6/5.2	28.6/5.2	11	50.8%

Table 2: Classification matrix for female and male datasets.

Female 100% ± 0 accuracy

	Control	*PCZ*
Control	6	0
PZC	0	6
No Class	0	0
Error	**0**	**0**

Male: 90% ± 22.4 accuracy

	Control	*PCZ*
Control	6	1
PZC	0	5
No Class	0	0
Error	**0**	**1**

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