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RESEARCH ARTICLE

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Proteomics analysis of zebrafish larvae exposed to 3,4-dichloroaniline using the fish embryo acute toxicity test

Leonardo R. Vieira[1] | Denise C. Hissa[2] | Terezinha Maria de Souza[3] | Chayenne A. Sá[1] | Joseph A. M. Evaristo[4] | Fábio C. S. Nogueira[4,5] | Ana F. U. Carvalho[2] | Davi F. Farias[1,6]

[1]Department of Biochemistry and Molecular Biology, Federal University of Ceara, Fortaleza, Brazil

[2]Department of Biology, Federal University of Ceara, Fortaleza, Brazil

[3]Department of Toxicogenomics, GROW School for Oncology and Developmental Oncology, Maastricht University, Maastricht, The Netherlands

[4]Laboratory of Proteomics, LADETEC, Institute of Chemistry, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil

[5]Proteomics Unit, Institute of Chemistry, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil

[6]Department of Molecular Biology, Federal University of Paraiba, João Pessoa, Brazil

Correspondence
Davi F. Farias, Department of Molecular Biology, Federal University of Paraiba, 58051-900 João Pessoa, Brazil.
Email: davi@dbm.ufpb.br

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Abstract

The zebrafish (*Danio rerio*) is a small teleost fish that is becoming increasingly popular in laboratories worldwide and several attributes have also placed the zebrafish under the spotlight of (eco)toxicological studies. Since the 1990s, international organizations such as ISO and OECD have published guidelines for the use of zebrafish in ecotoxicological assessment of environmental toxicants such as the Fish Embryo Acute Toxicity (FET) test, OECD n° 236 guideline. This protocol uses 3,4-dichloroaniline (DCA), an aniline pesticide whose toxicity to fish species at early life stages is well known, as a positive control. Despite its use, little is known about its molecular mechanisms, especially in the context of the FET test. Therefore, this study aimed to investigate such changes in zebrafish larvae exposed to DCA (4 mg/L) for 96 hours using gel-free proteomics. Twenty-four proteins detected in both groups were identified as significantly affected by DCA exposure, and, when considering group-specific entities, 48 proteins were exclusive to DCA (group-specific proteins) while 248 were only detected in the control group. Proteins modulated by DCA treatment were found to be involved in metabolic processes, especially lipids and hormone metabolism (eg, Apoa1 and Apoa1b and vitelogenins), as well as proteins important for developmental processes and organogenesis (eg, Myhc4, Acta2, Sncb, and Marcksb). The results presented here may therefore provide a better understanding of the relationships between molecular changes and phenotype in zebrafish larvae treated with DCA, the reference compound of the FET test.

KEYWORDS

chemical contaminants, endocrine disruptors, OECD FET test guideline, proteomics, reference compound

1 | INTRODUCTION

The zebrafish (*Danio rerio*) is a small teleost that is becoming increasingly popular in laboratories worldwide.[1] The low costs in rearing and production compared to mammals, large offspring, fast sexual maturation (at approximately 90 days of age), and small size are some of the general attributes that make zebrafish the first choice model organism in many biomedical and environmental studies.[2-4] This model has shown sensitivity to a wide variety of contaminants—for example, toxic heavy metals, endocrine disruptors, and organic pollutants[5]—indicating their suitability as a biological platform for environmental monitoring in risk assessment.

Since the 1990s, international organizations such as ISO and OECD have published guidelines for the use of zebrafish in (eco)

toxicological assessment of environmental toxicants.[6-8] These protocols are being continuously improved to provide more informative end points and to comply with ethical and economic issues regarding animal experimentation. For instance, the replacement of adult fish tests for the ones employing immature life stages (eg, embryos and larvae) has been strongly encouraged, resulting in the publication of a standardized version of the Fish Embryo Acute Toxicity (FET) test by OECD in 2013.[8] The FET test n° 236 has been regarded as a promising alternative to adult-based guidelines such as the OECD fish acute toxicity test n° 203,[9] being increasingly used for regulatory and research purposes.[10]

The FET test is suitable for a wide variety of substances, except for compounds with molecular mass larger than 3 kDa and those with toxicity specific to juvenile and adult life stages.[8] For the validation of the test, several parameters must be obeyed including the use of three different types of controls: negative (dilution water), internal (dilution water), and positive (3,4-dichloroaniline—DCA).[8] DCA, the reference compound in the FET test, is an aniline pesticide whose toxicity to fish species at early life stages is well documented.[11-14]

Despite its use as a toxic reference compound and status as a potentially hazardous environmental pollutant, very little is known about the molecular effects of DCA. Targeted studies have shown changes in expression of oxidative-stress related genes in exposed embryos.[15,16] A genome-wide investigation of DCA effects revealed that, in addition to oxidative stress, several genes involved in cytoskeleton structure and function, proteolysis, translation in mitochondria, response to hypoxia, muscle contraction, and cell cycle are also regulated.[17] However, whether these changes are consistently observed at the functional level, the translated proteins, remains unknown. Since the proteome, rather than the transcriptome, is closer to physiology,[18] investigation of the former 'ome provides better links between genotype and the observed phenotype.

Thus, we envisioned that a proteomic study could provide more information about the morphological changes in zebrafish larvae exposed to DCA and therefore understand how changes at the molecular level may be associated with the malformations that occur in these larvae when they are exposed to this molecule. This would add more information to the FET test and, therefore, a better understanding.

Using proteomics gel-free, we provide a global picture of protein expression in zebrafish larvae, hundreds of which have been shown to be modulated by DCA, focusing on biological processes and protein-protein interaction networks that may be affected by the treatment and its relationship with apical endpoints. These data may therefore contribute to improving the robustness of the FET test through a better understanding of the effects of this substance. In this work, we attempted to relate the changes in the proteome of zebrafish larvae exposed to DCA with the malformations observed in them.

2 | MATERIALS AND METHODS

2.1 | Maintenance and rearing of zebrafish

A wild-type strain of zebrafish (*D. rerio*) was reared in the animal facility from the Department of Biology (The Federal University of Ceará, Fortaleza, Brazil). The strain was cultured at 26 ± 1°C under a 14:10 hour light:dark photoperiod. Water pH was maintained from 6.8 to 7.9, with dissolved oxygen ranging from 5 to 7 mg/L and water hardness of 0.45 mg $CaCO_3$ per liter. Fish were fed daily with commercial food (Topical Gran Discus, Sarandi, Brazil) and *Artemia* sp. nauplii, being also monitored for abnormal behavior or disease development.

To obtain embryos, male and female specimens (3:1 ratio) were placed overnight in spawning tanks containing egg traps on the day prior to testing. One hour after the beginning of the light cycle, fertilized eggs were collected and washed for subsequent use in the FET test.

All experiments conducted with zebrafish embryos in this study were approved by the Ethics Commission in Animal Use in Research (CEUA), certified by protocol number 79/16 by the Federal University of Ceará.

2.2 | FET test

The FET test was conducted with DCA according to OECD's guideline number 236.[8] Twenty fertilized eggs were placed in 24-well plates (one embryo per well) and exposed to 4.0 mg/L DCA. This concentration is indicated by the guideline as positive control and test validation. Four eggs, exposed to dilution water only, were used as internal plate controls. An additional plate containing embryos only in dilution water was also incubated. Eggs with up to 3 hpf (hours post-fertilization) of age were exposed to DCA for 96 hours and embryos were analyzed every 24 hours for the apical endpoints (eg, g coagulation, lack of somite formation, lack of detachment of the tail-bud from the yolk sac, and lack of heartbeat). Controls and DCA solutions were also renewed after 24 hours. Observations were performed in a stereo microscope (×80 magnification) and photographed (Nikon). After 96 hours, surviving larvae were analyzed and euthanized with eugenol and discarded.

2.3 | Exposure of embryos to DCA for proteomic analysis

For proteomic analysis, 20 embryos of zebrafish up to 3 hpf were placed into beakers containing 40 mL DCA solution (4.0 mg/L) or dilution water. Solutions were carefully renewed every 24 hours to avoid significant variation in the medium. Dead larvae spotted in the process were removed and discarded. After 96 hours of exposure, surviving larvae were rapidly frozen by immersion in liquid nitrogen and stored at −80°C until analyses. Each experimental group (test and control) comprised three replicates.

2.4 | Protein extraction

For protein extraction, individual from all replicates were merged per experimental group. Control and test larvae were dry-frozen and subsequently resuspended in 200 μL of a 7 M urea/2 M thiourea

containing 1% sodium deoxycholate for total protein extraction. Extraction was performed under constant stirring for 2 hours at room temperature. Samples were then centrifuged at 10000*g* for 30 minutes at 25°C. To the supernatant was added 1 mL of cold acetone/10% trichloroacetic acid solution (TCA), which was kept at −20°C. After 16 hours, these were centrifuged at 15000*g* for 30 minutes at 4°C. The remaining pellet was washed three times with cold acetone to remove any residual TCA. The pellet was dried at room temperature and stored at −80°C until further analyses.

2.5 | Sample preparation for LC-MS/MS

In this study we used a bottom-up gel free approach. For this, 300 μg protein were resuspended in 45 μL of a 7 M urea/2 M solution and reduced with 100 mM dithiothreitol (final concentration of 10 mM) at 35°C for 1 hour under constant stirring (500 RPM). Samples were then alkylated by adding 40 mM iodoacetamide, which was further homogenized for 30 minutes at 35°C (500 RPM) in the dark. To the final volume (60 μL), 480 μL water were added to dilute the urea (<1 M). The proteins were then hydrolyzed with 3.0 μg trypsin (0.1 mg/L, Promega). The process of digestion occurred for 16 hours at 35°C (500 RPM), after which was stopped with the addition of trifluoroacetic acid (TFA) at a final concentration of 0.1%. Samples were desalinated and concentrated through reverse phase chromatography with a resin column POROS 20 R2 (Applied Biosystems), eluted with 60% acetonitrile/0.05% TFA. The peptides were dried, resuspended in 0.1% formic acid, quantified (Qubit protein assay kit, Thermo Scientific), and analyzed with an Easy1000-nano liquid chromatography system coupled to an nESI-Q-Exactive Plus mass spectrometer (both from Thermo Scientific). Three biological replicates were analyzed twice (two technical replicates).

Two micrograms of peptides were analyzed in technical duplicate after 2 hours of gradient (5% to 40% B/100 minutes; 40% to 95% B/3 minutes; 95% B/17 minutes). nanoLC solvent A consisted of (95% H_2O/5% acetonitrile [ACN]/0.1% formic acid) and solvent B of (95% ACN/5% H2O/0.1% formic acid). Trap-column length was 3 cm with 200 μm ID (5 μm spheres-Reprosil Pur C18, Dr. Maish) and analytical column of 20 cm and 75 μm ID (3 μm spheres—Reprosil Pur C18, Dr. Maish). Q-Exactive Plus in FullScan-DDA MS2 mode used a dynamic exclusion list of 45 seconds and spray voltage at 2.7 kV. Full scan was acquired at a resolution of 70 000 at *m/z* 200, with an *m/z* range of 350 to 2000, AGC of 1×10^6, and injection time of 50 milliseconds. Selection of the 20 most intense ions for HCD fragmentation used a normalized collision energy of 30, precursor isolation window of *m/z* 1.2 and 0.5 offset, a resolution of 17 500 at *m/z* 200, AGC at 5×10^5, and injection time of 100 milliseconds.

2.6 | MS data analyses

Data obtained from MS were analyzed against the reference proteome of *D. rerio* available in UniProt (July 2018). A total of 46 900 sequences were downloaded. PatternLab for Proteomics 4.0[19] was used to generate a target-decoy data set by compiling subsets of sequences, including 127 common contaminants in MS and an inverted version for each sequence. Further analyses were conducted according to the steps described on the software guideline. In summary, the match criterion used in this study comprised semi-tryptic peptides with the parameters carbamidomethylation cysteines and oxidation of methionine as fixed and variable modifications, respectively. Comet, a module integrated to PatternLab, was used to match MS patterns to the stored sequences. The Search Engine Processor (SEPro) was used to validate the correspondences of peptide sequence.[20] A false discovery rate (FDR) cutoff up to 0.01 at peptide and protein level was applied considering the number of decoys, with the minimum amino acid sequence set to six. A minimum score of four for "spectral count" and two for "peptide count" was also applied.[19,21]

2.7 | Label-free quantification

Quantitative proteomic analysis was performed according to the normalized spectral abundance factors (NSAF) provided by the SEPro engine in the previous step.[22] The TFold module was used to evaluate the levels of differentially expressed proteins (DEPs).[19] This module is based on a theoretical FDR estimation[23] that maximizes the number of identifications that satisfy both a fold change cutoff and a stringency criterion (aiming to detect lowly abundant proteins that may increase the rate of false positives).

2.8 | Functional analysis of DEPs: gene ontology (GO) annotation, pathway, and network analysis

The tool Blast2GO (blast2go.com)[24] was used to classify the DEPs identified according to the current GO annotation (March 2018; geneontology.org).[25]

Furthermore, DEPs were mapped into KEGG pathways available for zebrafish using the KEGG Mapper tool (http://www.genome.jp/kegg).[26]

Network analysis was performed using the Search Tool for the Retrieval of Interacting Genes/Proteins available on STRING (string-db.org)[27] to obtain protein-protein interactions available for zebrafish. Interactions were obtained by using group-specific and DEPs as input and applying a high-confidence filter (higher than 0.7). Retrieved interactions were then uploaded into Cytoscape (v. 2.8.1)[28] to build a network comprised of proteins (nodes) and their interactions (edges connecting nodes). Edgeless nodes were removed from the final network for clarity.

3 | RESULTS AND DISCUSSION

In order to obtain novel insights into the molecular mechanisms underlying the OECD's FET test (n. 236), this study aimed to

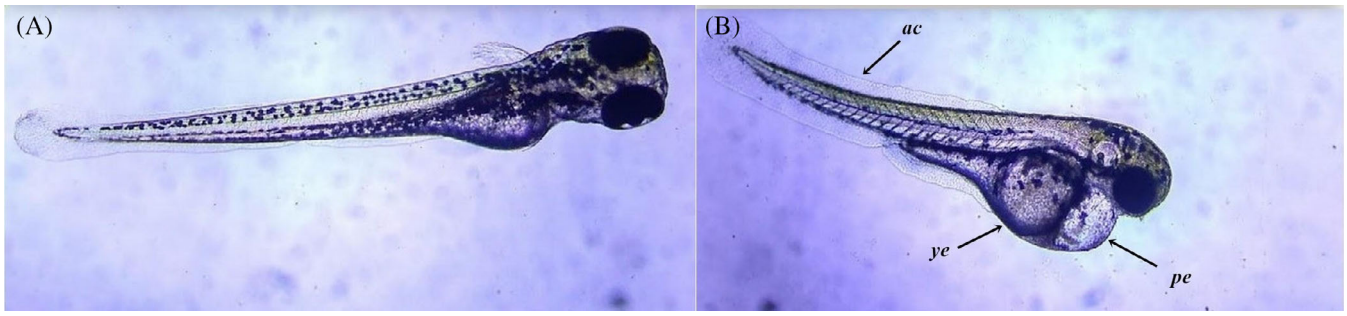

FIGURE 1 Morphology of larvae after 96 hours of exposure to, A, negative control (water) and, B, 3,4-dichloroaniline (DCA), conducted in accordance to the OECD FET test guideline. *ac*: abnormal curvature of the spine; *ye*: yolk sac edema; *pe*: pericardial edema. FET, Fish Embryo Acute Toxicity [Color figure can be viewed at wileyonlinelibrary.com]

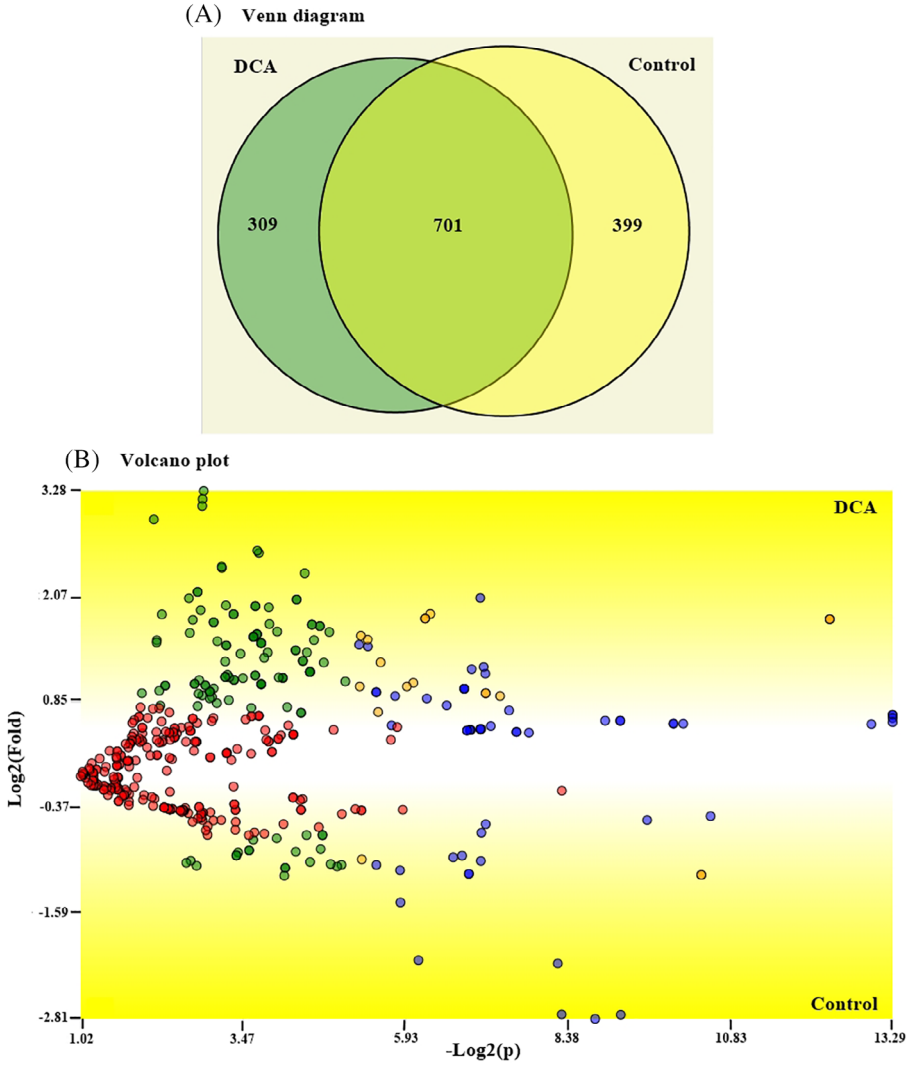

FIGURE 2 A, Venn diagram of zebrafish proteins treated with 3,4-dichloroaniline and water (negative control). Seven hundred one proteins were shared by both groups, while 309 and 399 were specific to 3,4-dichloroaniline and control, respectively. B, Volcano plot and quantitative analysis of proteins shared by both treatments (−log2 *P*-value in the x-axis and log2 fold change [FC] in the y-axis). Red dots indicate proteins that do not meet the fold change and false discovery rate (FDR) criteria established in this study. Green dots indicate those that meet the FC cutoff but not FDR. Orange dots indicate proteins that meet the FC and the FDR criteria, but as abundant proteins, need more experiments to confirm significance of differential expression. Blue dots indicate proteins that meet both FC and FDR cutoffs (56 after considering redundancy) [Color figure can be viewed at wileyonlinelibrary.com]

investigate alterations in the proteome of zebrafish larvae induced by the standard control recommended by that protocol, DCA. For that, we analyzed the total proteome of larvae following a 96-hour exposure period to the agent using label-free proteomics (LC-MS/MS).

For the toxicity test, water temperature, dissolved oxygen, eclosion, and survival rate were all in accordance with OECD's parameters.[8] Among the four endpoints indicative of lethality as described by the FET test, only egg coagulation was not observed in larvae exposed to DCA in this study. Non-detachment of tail, lack of somite

TABLE 1 Differentially expressed proteins in (upregulated and downregulated) in larvae exposed to 3,4-dichloroaniline in comparison to negative control (water)

UniProt ID	Protein name	Log_2 fold change	Function
Upregulated proteins*			
Q1LWN2	Vitellogenin 1	1.53	Response to xenobiotic stimulus; lipid transporter activity; antioxidant activity
A0A2R8Q549	Vitellogenin 2	1.40	Lipid transporter activity
A0A2R8RN02	Vitellogenin 4	1.45	Lipid transporter activity; protein binding
Q4QRF3	Vitellogenin 5	1.85	Lipid transporter activity
F1QV15	Vitellogenin 6	1.51	Lipid transporter activity
F1R2T3	Vitellogenin 7	1.44	Lipid transporter activity
A0A2R8Q2W1	Serine/arginine-rich-splicing factor 1A	1.94	mRNA processing; nucleic acid binding
F8W3L1	Actin, alpha 2 (smooth muscle)	1.48	Heart contraction; blood circulation
A0A2R8QEN9	Heat shock cognate 71 kDa protein	2.25	Protection of the proteome from stress; folding and transport of newly synthesized polypeptides; activation of proteolysis of misfolded proteins
Q8JHI0	Solute carrier family 25	2.80	ATP:ADP antiporter activity; regulation of mitochondrial membrane permeability
Q6NYV3	H1 histone family, member 0 (Zgc:65861)	2.84	DNA binding; nucleosome assembly
E9QIH5	Glyceraldehyde-3-phosphate dehydrogenase	1.88	Microtubule binding; glucose metabolic process; glycolytic process; neuron apoptotic process; microtubule cytoskeleton organization
B8JL29	High mobility group box 2a	4.13	DNA binding
Downregulated proteins*			
Q1LXJ7	Type I cytokeratin, enveloping layer,-like	−1.38	Structural molecule activity
F1QAF1	Myosin heavy chain 4	−1.57	Microtubule-based movement; microtubule motor activity; actin filament binding
F1QK60	Keratin 4	−1.97	Structural molecule activity
Q8JH71	Fructose-bisphosphate aldolase B (EC 4.1.2.13) (Liver-type aldolase)	−2.19	Glycolytic process; fructose-bisphosphate aldolase activity
A2BHA3	Creatine kinase, muscle a	−1.47	ATP binding; kinase activity
Q6NWH2	MARCKS-like 1b	−4.49	Calmodulin binding; cartilage development
Q7SX92	Beta-synuclein	−6.76	Larval locomotory behavior; dopaminergic neuron differentiation
O42363	Apolipoprotein A-I	−2.04	Cholesterol transporter activity; cholesterol transporter activity
A0A0R4IKF0	Apolipoprotein A-Ib	−4.37	Lipoprotein metabolic process; lipid binding
F6NXD5	Myristoylated alanine-rich protein kinase C substrate b	−2.75	Brain development; respiratory system development; retina layer formation
Q66I37	Nuclear casein kinase and cyclin-dependent kinase substrate 1a	−2.13	Kinase activity; phosphorylation

*P-value <.01.

formation, and the absence of heartbeat were detected after 48 hours of exposure. At the end of exposure (96 hours), 91.7% (±7.6) of embryos presented at least one of the aforementioned endpoints. This value is also found to be similar to that conducted by Busquet and coworkers (2014) with the objective of measuring intra- and inter-laboratory reproducibility of the FET test.[29] Furthermore, in our study, mortality rates were below 10% in the negative control which were in accordance with OECD's guidelines.

In addition to the four standard lethality endpoints, we also observed the presence of other deformities such as the formation of pericardial edema, abnormal curvature of the spine, and yolk sac edema (Figure 1). Voelker and coworkers (2008) have also reported the occurrence of additional malformations in DCA-exposed embryos after 50 hours, including discoloration, reduction in head and eye sizes, shrunk tail, as well as yolk sac edema and abnormal spine curvature.[3]

TABLE 2

Uniprot ID	Protein name	Function
E9QIR9	60S ribosomal protein L10	Structural constituent of ribosome; embryonic brain development; translation
Q6PC14	60S ribosomal protein L23	Translation
D1GJ56	Actinin alpha 3a	Calcium ion binding; actin binding
Q5CZR5	Aerolysin-like protein	Protein homooligomerization; disaccharide binding
O42364	Apolipoprotein Eb	Amyloid-beta binding; cholesterol transporter activity; neuron projection regeneration; negative regulation of neuron apoptotic process
B2CZC0	Calcium-transporting ATPase	Calcium ion transmembrane transport
P79722	Cathepsin Lb	Proteolysis; proteolysis involved in cellular protein catabolic process
A0A0R4IMA3	Collagen, type II, alpha 1a	Notochord development; embryonic skeletal system development
A7E2K5	Crystallin, beta B1,-like 2	NF
Q8UW07	Cytochrome P450	Cellular response to xenobiotic stimulus
E7FE90	Fetuin B	Cysteine-type endopeptidase inhibitor activity
A2CEW3	Fibronectin 1b	Somitogenesis; atrioventricular valve morphogenesis; heart field specification
Q5MJ86	Glyceraldehyde-3-phosphate dehydrogenase 2	Glycolytic process; glucose metabolic process
A0A286YAP4	Heat shock protein 90, beta	Response to hypoxia
X1WFI9	High mobility group box 1b	DNA binding
A8E588	Im:6910535 (Zgc:174154)	Proteolysis involved in cellular protein catabolic process
A0A0R4IUQ9	Interleukin enhancer-binding factor 3b	Regulation of transcription, DNA-templated; transcription, DNA-templated
A0A140LGS3	Jacalin 1	NF
Q5RJ97	Lamin B receptor	Cell migration involved in gastrulation; sterol biosynthetic process
Q5NJJ5	Matrilin-4	Calcium ion binding; magnesium ion binding
A7MCR8	MGC174857 protein (Si:dkey-26 g8.5)	Proteolysis involved in cellular protein catabolic process
Q7ZUN6	Phosphoribosylaminoimidazole carboxylase	Pigmentation; chordate embryonic development; camera-type eye development
A0A0R4IRG2	Procollagen, type V, alpha 1	Extracellular matrix structural constituent
Q6DHS3	Ribosomal protein L15	Cytoplasmic translation; RNA binding
Q6DGL0	Ribosomal protein L18	RNA binding; translation
Q5BJJ2	Ribosomal protein L3	Exocrine pancreas development; translation; RNA binding
Q7ZUI0	Ribosomal protein L5a	Chordate embryonic development; translation
A0A286YAR2	Ribosomal protein L5b	5S rRNA binding; translation
Q567N5	Ribosomal protein L6	Chordate embryonic development; pancreas development
Q6NYA0	Serine/arginine-rich splicing factor 1B	RNA splicing; RNA binding
E9QHD6	Serine/arginine-rich-splicing factor 2a	Regulation of RNA splicing; RNA binding

(Continues)

TABLE 2 (Continued)

Uniprot ID	Protein name	Function
Q6NZT9	Serine/arginine-rich-splicing factor 2b	Regulation of RNA splicing; RNA binding
B8JLR6	Si:ch211-251b21.1	Ion transport; extracellularly glutamate-gated ion channel activity
E9QBE2	Si:dkey-239j18.2	Proteolysis involved in cellular protein catabolic process
E7F8Y7	Si:dkey-239j18.3	Proteolysis involved in cellular protein catabolic process
X1WB76	Si:dkey-269i1.4	Proteolysis; peptidase activity; hydrolase activity
A0A2R8Q790	Si:dkey-26 g8.4	Proteolysis; peptidase activity; hydrolase activity
F1QSR3	Tenascin N	Regulation of cell proliferation; cell adhesion
Q7ZU99	Transitional endoplasmic reticulum ATPase	Chordate embryonic development; autophagosome maturation; autophagy; cellular response to DNA damage stimulus
Q6DHP2	Troponin I type 2b (skeletal, fast)	Cardiac muscle contraction; skeletal muscle contraction
E9QIW4	Troponin T type 3b (skeletal, fast)	Myofibril assembly; regulation of muscle contraction; sarcomere organization
Q6GQM1	Tubulin alpha chain	Microtubule-based process
F1QKY8	Tubulin beta chain	Microtubule-based process
A0A2R8Q5Z6	Uncharacterized protein	NF
A0A2R8QNF0	Uncharacterized protein	NF
Q29RA2	Zgc:136908	Hydrolase activity
F1R8Y0	Zgc:174153	Proteolysis involved in cellular protein catabolic process
F1Q9G9	Zgc:66156	Microtubule-based movement

Abbreviation: NF, not found.

Concerning DCA-induced alterations in the proteome of treated larvae using gel-free and label-free proteomics, we identified 1409 proteins (employing FDR < 0.01 and taking redundancy into consideration). This number is similar to other protein-level investigations (albeit at baseline) performed previously in zebrafish.[30] Overall, 701 proteins were shared by both groups investigated (Figure 2A), while 309 and 399 were exclusive to the DCA and control groups, respectively. After considering expression in at least two of the three replicates and removal of redundant entities, these lists were decreased to 48 group-specific entities exclusive to DCA, while 248 were only detected in the control group. Fifty six DEPs were identified as significantly affected by DCA exposure, when using the same filtering (appearance in two of three replicates and redundancy entities removal) process, this list was decreased to 24 proteins (Table 1). A list of proteins only found in the DCA group can be found in Table 2. A volcano plot with the quantitative analyses performed can be found in Figure 2B. A complete list of these proteins can also be visualized in Tables S1-S3.

To obtain insights into the biological functions of DEPs identified, we categorized DEPs and group-specific proteins based on their GO classifications (biological process, molecular function, and cellular component) using Blast2GO. Categorization based on biological process of upregulated proteins showed that those are distributed in 18 classes, including "response to estradiol," "lipid transport," "cellular oxidant detoxification," "response to xenobiotic stimulus," and "cellular response to estrogen stimulus" (Figure S1A), while downregulated proteins were mapped to 18 classes, including phosphorylation and cell development processes (Figure S1B). Upregulated proteins were further found to integrate 14 classes of GO cellular components and 15 classes of molecular function (Figures S2A and S3A). DEPs repressed were categorized into 7 classes of cellular component and 12 classes of molecular function (Figures 2B and 3B). Proteins exclusive to DCA revealed that they are distributed in 24 categories such as proteolysis, organ and embryonic development, and metabolic process (Figure S4). Classification by cellular component and molecular function (Figure S4) identified 9 and 10 categories, respectively.

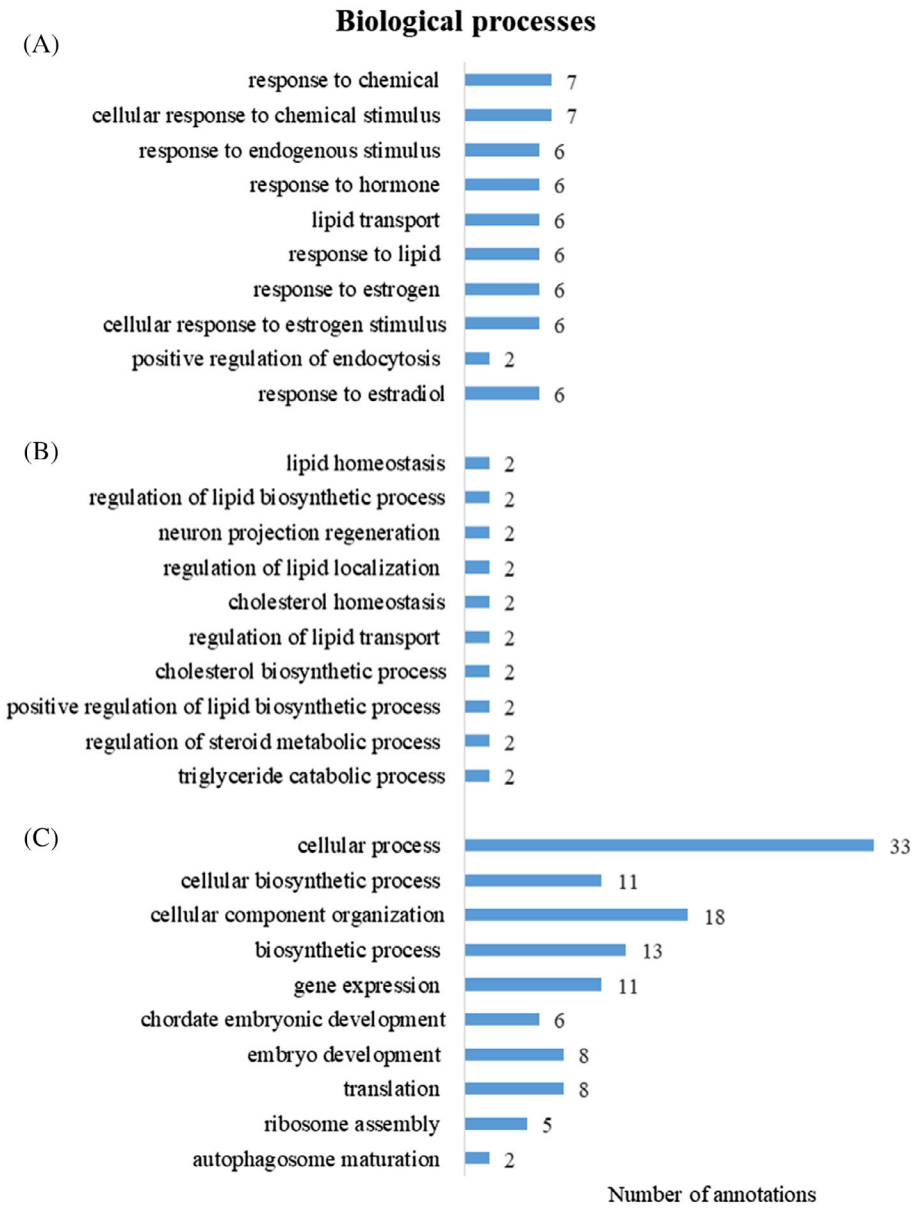

FIGURE 3 Enrichment analysis of gene ontology terms of proteins, A, upregulated and, B, downregulated in relation to negative control and, C, proteins found only in 3,4-dichloroaniline-treated zebrafish [Color figure can be viewed at wileyonlinelibrary.com]

To estimate the significance of blast2GO classifications, we further performed GO enrichment analysis. A total of 19 biological processes were enriched with proteins upregulated by DCA treatment (Figure 3A). These include hormone-related pathways (eg, "cellular response to estrogen stimulus," "response to estrogen," and "response to hormone,") lipid metabolism ("lipid transport," "lipid localization," and "lipid transporter activity"), as well as response to exogenous substances ("response to chemical," "cellular response to chemical stimulus," and "response to chemicals"). Interestingly, the overrepresentation of hormone-related pathways is in line with previous reports that show DCA as an endocrine disrupting chemical for both wildlife and humans.[31]

The overrepresentation of lipid-related pathways (Figure 3B) was also found in proteins identified as downregulated (eg, "lipid homeostasis," "regulation of lipid biosynthetic process," "glycerolipid catabolic process," "triglyceride catabolic process," "regulation of steroid metabolic process," and "positive regulation of lipid biosynthetic process"). This overrepresentation (regardless direction of expression of the component proteins) was found to be mainly due to the altered expression of apolipoproteins and vitellogenins, important in lipid transportation[32] and essential for mobilization of lipids from the yolk sac during the first 4 days of life to ensure growth and survival.[33] Two apolipoproteins (Apo1a and Apo1b) were detected as downregulated by DCA. Previous studies have shown the modulation of apolipoproteins in zebrafish in response to chemical stressors[34-36]; it has been suggested that they may have important roles in developmental toxicity, but the exact mechanisms remain to be elucidated. A complete description of the processes enriched by proteins from each group can be found in Tables S4-S6.

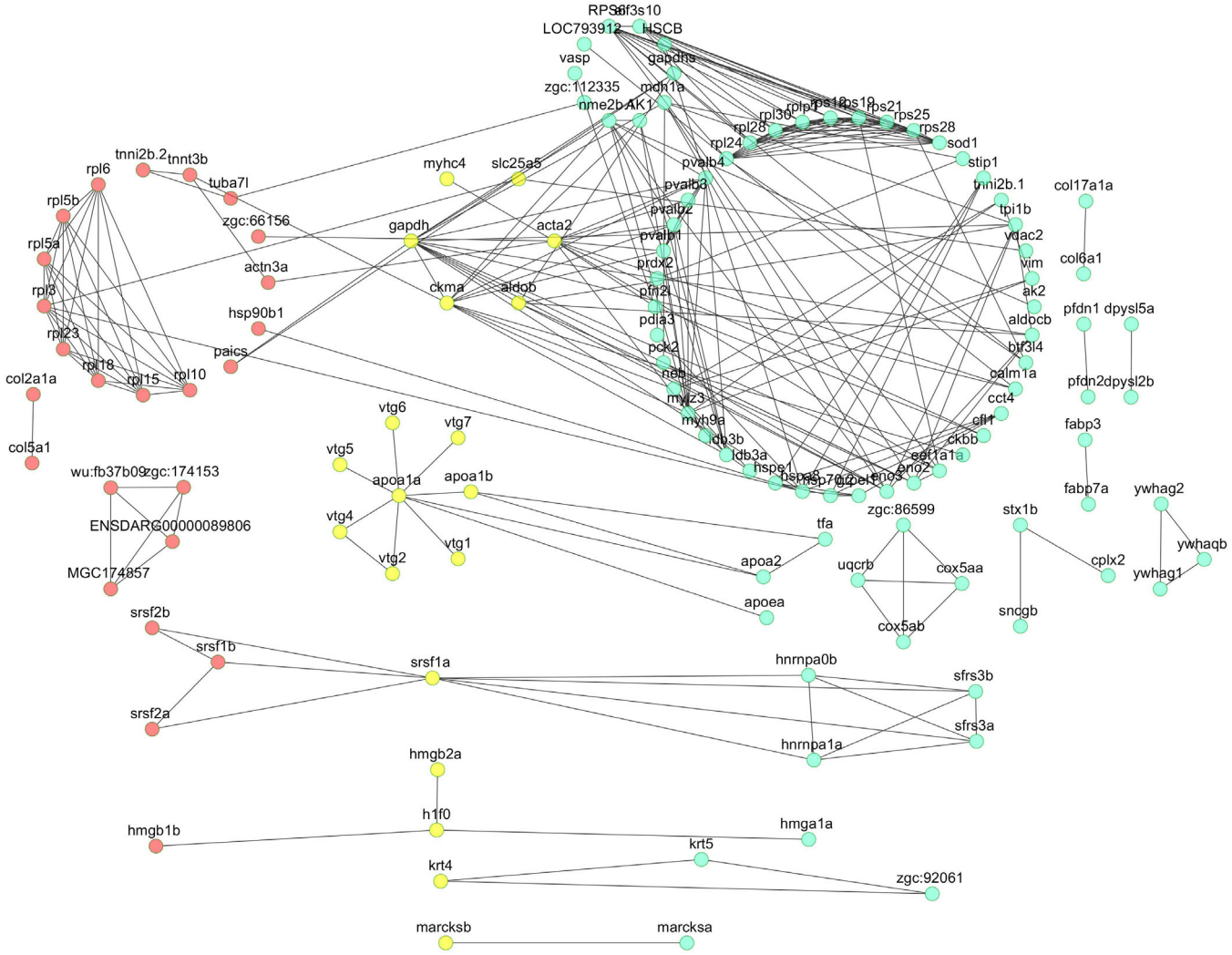

FIGURE 4 Protein interaction network constructed using STRING v10.5 to (i) proteins only found in 3,4-dichloroaniline (red nodes), (ii) differentially expressed proteins (yellow nodes), and (iii) proteins only found in water (blue nodes) [Color figure can be viewed at wileyonlinelibrary.com]

Furthermore, vitellogenins, identified as upregulated in this study, are often modulated in zebrafish larvae exposed to chemical stressors.[37] More importantly, they are used as biomarkers of environmental water pollution due to estrogenic substances.[38] Previous quantitative structure-activity relationship simulations with TCAB, a major DCA metabolite, have indicated that triggering of ERβ, mineralocorticoid, glucocorticoid, and progesterone receptor activities are expected.[39] Taken together, these findings strengthen the evidence of the endocrine disrupting effects of DCA and its alterations at the molecular level.

When considering group-specific proteins, we found 43 and 86 biological processes exclusive to DCA (Figure 3C) and control larvae, respectively. Interesting processes from proteins found in DCA only included "response to misfolded protein," "chordate embryonic development," and "embryo development." Proteins detected exclusively in control larvae were mainly related to metabolic processes and development ("glycolytic process," "aerobic respiration," "animal organ development," and "system development"). A complete description of the processes enriched by proteins from each group can be found in Tables S4-S6.

DEPs were also mapped to KEGG pathways in order to increase the specificity of biological processes affected by treatment with DCA. Proteins such as solute carrier family 25 (Slc25a5) were found in mitochondrial pathways related to necroptosis, cellular senescence, and calcium signaling (Table S7). Slc25a5 is part of a group of transmembrane proteins involved in transport of several substances such as drugs, toxins, cations, and anions.[40] Analysis of proteins found only in DCA-treated larvae showed that these entities were mapped to 21 different pathways (Table S8), including autophagy, necroptosis, base excision repair, apoptosis, phagosome, adrenergic signaling in cardiomyocytes, ribosome, spliceosome, and metabolism of xenobiotics cytochrome P450. These findings not only corroborate findings

from GO analyses but also point toward more specific processes indicative of toxicity.

On the other hand, mapping of proteins exclusive to larvae from the negative control group resulted in identification of 50 pathways (-Table S9), including several processes involved in heart function and development (eg, cardiac muscle contraction, vascular smooth muscle contraction, neuroactive ligand-receptor interaction, adrenergic signaling in cardiomyocytes) and metabolism (eg, pentose phosphate pathway, glycolysis/gluconeogenesis, pentose phosphate pathway, biosynthesis of amino acids, and carbon metabolism). This indicates, therefore, that DCA may play a role in completely repressing the activity of certain proteins necessary to normal development and survival.

Other interesting deregulated proteins include actin, alpha 2 (smooth muscle) (Acta2), a protein directly related to heart function,[41] detected as upregulated. Actins are highly conserved among vertebrates,[42] and mutations in the *Acta2* gene in humans are linked to promotion of coronary diseases and obstruction/increase of arteries.[43] This protein may therefore be a putative marker for DCA-induced alterations in zebrafish heart function (decrease in heartbeat frequency). Furthermore, beta-synuclein (Sncb) was detected as downregulated; this protein is directly involved in larval locomotor behavior.[44,45] Beta synucleins are necessary for normal development of the dopaminergic system of zebrafish,[46] and DCA-induced downregulation of this protein may be involved in the observed reduction of locomotion in treated larvae.[44] Marcksb, modulated by DCA, has been shown to be essential to the development of gills in zebrafish.[47]

Finally, to capture how a potential network of interactions among these proteins, we used the String database to build a protein interaction network (PIN) based on group-specific proteins and DEPs. PINs are important because they are able to capture properties of biological processes and help to understand and model events leading to a diseased phenotype. Figure 4 summarizes these interactions among (a) proteins only found in DCA (red nodes), (b) DEPs (yellow nodes), and (c) proteins only found in water (blue nodes). This PIN shows the interactions not only between proteins such as apolipoproteins and vitellogenins but also between structural proteins such as Acta2 and metabolism-related proteins such as fructose-bisphosphate aldolase B (Aldob) and glyceraldehyde-3-phosphate dehydrogenase (Gapdh) (both involved in energetic metabolism), suggesting a crosstalk among different pathways. It also highlights a cluster of ribosomal proteins (eg, ribosomal Protein L6), which have been shown to play important roles in normal zebrafish development, pointing toward an association between deregulated expression of ribosomal proteins and developmental abnormalities.[48] Overall, this PIN summarizes a network of interactions contrasting normal and disturbed states, which may help further investigations assessing the sequence of events leading to DCA-induced toxicity.

4 | CONCLUSION

In conclusion, our study has identified molecular-level alterations linked to exposure to DCA, a substance used as reference compound in OECD's FET test. We identified changes in the proteome using label-/gel-free proteomics, pinpointing biological processes, pathways, and potential networks involved in DCA-induced embryonic lethality, with weight-of-evidence spanning across different databases (Blast2GO, GO enrichment, and KEGG pathway membership) indicative of processes suggesting the endocrine disruptive and teratogenic potential, especially in heart, of the substance DCA. We show how these changes may be related to observed apical endpoints, which may help to better understanding of molecular phenotypic relationships and serve as potential molecular markers of these toxic endpoints, aiding future investigations on the potential effects in human populations as well.

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ORCID

Davi F. Farias https://orcid.org/0000-0001-5438-1919

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