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# Assessing the effects of an acute exposure to worst-case concentration of Cry proteins on zebrafish using the embryotoxicity test and proteomics analysis

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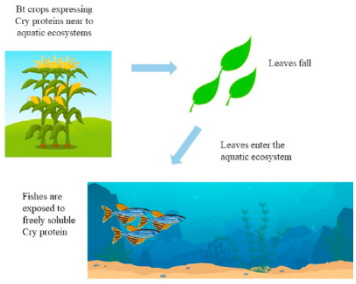
## HIGHLIGHTS

- Cry1C, Cry1F and Cry1Ab proteins did not induce malformations or mortality in zebrafish embryos/larvae.
- Cry proteins did not cause changes in enzyme biomarkers of zebrafish larvae.
- Cry proteins induced minor disturbances in the proteome of zebrafish larvae.
- Cry1C, Cry1F and Cry1Ab proteins have no deleterious effects on fish.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Cry1C, Cry1F and Cry1Ab are insecticidal proteins from *Bacillus thuringiensis* (*Bt*) which are expressed in transgenic crops. Given the entry of these proteins into aquatic environments, it is relevant to evaluate their impacts on aquatic organisms. In this work, we sought to evaluate the effects of Cry1C, Cry1F and Cry1Ab on zebrafish embryos and larvae of a predicted worst-case scenario concentration of these proteins (set to 1.1 mg/L). For that, we coupled a traditional toxicity approach (the zebrafish embryotoxicity test and dosage of enzymatic biomarkers) to gel free proteomics analysis. At the concentration tested, these proteins did not cause adverse effects in the zebrafish early life stages, either by verifying phenotypic endpoints of toxicity or alterations in representative enzymatic biomarkers (catalase, glutathione-S-tranferase and lactate-dehydrogenase). At the molecular level, the Cry proteins tested lead to very small changes in the proteome of zebrafish larvae. In a global way, these proteins upregulated the expression of vitellogenins. Besides that, Cry1C e Cry1F deregulated heterogeneous nuclear ribonucleoproteins (Hnrnpa0l and Hnrnpaba, respectively), implicated in mRNA processing and gene

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regulation. Overall, these data lead to the conclusion that Cry1C, Cry1F and Cry1Ab proteins, even at a very high concentration, have limited effects in the early stages of zebrafish life.



## 1. Introduction

Cry1C, Cry1F and Cry1Ab are proteins expressed in *Bacillus thuringiensis* (*Bt*) transgenic crops due to their activity against lepidopteran insects (Baktavachalam et al., 2015; Gao et al., 2018; Yang et al., 2016). The consolidation of *Bt*-plant technology over the last three decades is due in part to the high selective toxicity and efficacy of the Cry proteins against certain insect orders (Palma et al., 2014). This property directly promotes a more cost-efficient pest control than that observed in the use of synthetic chemical insecticides by reducing the impacts on non-target organisms and ecological relationships (Chattopadhyay et al., 2017; Peralta and Palma, 2017).

As with other Cry proteins, studies indicate that Cry1C, Cr1F and Cry1Ab do not represent a considerable risk to the environment (CERA (Center for Environmental Risk Assessment)I.R.F., 2011; EFSA GMO Panel, 2012; Baktavachalam et al., 2015; Koch et al., 2015; Chen et al., 2018; Gao et al., 2018; Huang et al., 2019). Although most of the data comes from experiments with terrestrial organisms, many efforts have been made to assess the effects of these proteins on aquatic life which includes vertebrates such as fishes. So far, it is known that rainbow trout (*Onchorhynchus mykiss*) and catfish (*Silurus glanis*) fed with diets containing Cry1F and Cry1Ab, respectively, did not show any sign of toxicity, and the $LD_{50}$ of Cry1F for *O. mykiss* is greater than 100 mg a. i./kg (US EPA Office of Pesticide Programs, 2010). In addition, Grisolia et al. (2009) reported that zebrafish larvae treated with up to 25 mg/L of Cry1Ab did not show no sign of toxicity. Furthermore, Gao et al. (2018) recently reported that the toxin Cry1C (10 mg/L) does not cause any deleterious effect in zebrafish larvae. In general, studies carried out with other Cry proteins in fish (mainly zebrafish) have suggested that these molecules are not harmful to these vertebrates (Grisolia et al., 2009a, 2009b; Sissener et al., 2011, 2010; Sanden et al., 2013; Gao et al., 2018).

The relevance of studying the effects of *Bt* products on aquatic organisms relies on the fact that these animals play important roles in ecological relationships and the food chain in both aquatic and terrestrial environments, keeping the balance of aquatic pH and the consumption of pest insects, plant matter and algae (Hodson, 1997). Furthermore, aquatic environments are the final destination of most contaminants, and Cry proteins could also reach these ecosystems (Strain and Lydy, 2015; Tank et al., 2010). In fact, aquatic animals can be exposed to *Bt*-crop Cry proteins via solubilization of these molecules in aquatic environments (Carstens et al., 2012; Venter and Bøhn, 2016), even though it is known that they have low environmental persistence (Koch et al., 2015; Venter and Bøhn, 2016). By using the US EPA standard pond model (a mathematical prediction model) for worst-case scenario assumptions, Carstens et al. (2012) estimated that the concentration of representative Cry proteins derived from *Bt* corn biomass as freely soluble protein in the water column could reach a maximum of 1.125 mg/L.

Toxicity assays with fish are usually required to evaluate the environmental hazard of drugs, bioinsecticides, biomolecules derived from plants and other chemicals (Busquet et al., 2014; OECD, 2013; Bambino and Chu, 2017). In 2013, the OECD launched the fish embryo acute toxicity (FET test no. 236) test, also called zebrafish embryotoxicity test (ZET), to evaluate toxicity of chemicals in the zebrafish early stages development (OECD, 2013). The general principles of this guideline have already been adopted in some investigations to assess the toxicity of *Bt* Cry proteins in zebrafish (Gao et al., 2018; Grisolia et al., 2009).

Grisolia et al. (2009) tested the embryotoxicity of Cry1Aa, Cry1Ab, Cry1Ac and Cry2A proteins in zebrafish, using concentrations ranging from 25 to 150 mg/L. At the lowest concentrations tested, all these proteins caused embryotoxicity and developmental delay in the zebrafish early life stages. In turn, Gao et al. (2018) used a modified version of the FET test to assess the effects of Cry1C and Cry2A, performing exposures to concentrations of 0.1, 1 and 10 mg/L of both proteins. In addition to verify the morphological endpoints of lethality recommended by the guideline, the authors analyzed the expression of six genes related to oxidative stress and apoptosis, as well as evaluated the activity of some enzyme biomarkers (superoxide dismutase and catalase) and levels of lipid peroxidation. The results showed no deleterious effects of the Cry proteins tested on zebrafish embryos or larvae, even at the highest concentrations. It is important to note that most of the concentrations tested (>10 mg/L) in these studies are many times higher than those environmentally found (Strain and Lydy, 2015; Tank et al., 2010) or even those assumed as the worst-case scenario of environmental exposure to Cry proteins (Carstens et al., 2012).

Traditional ecotoxicity tests such as the FET test provide important information within their purposes (Sherry, 2003). These tests are designed to be performed in laboratory conditions, with high reproducibility and based on short-term exposures (1–28 days) of model organisms to high concentrations of (potential) toxicants and verification of lethality endpoints (e.g. death, reproductive failure) (Sherry, 2003; Villeneuve and Garcia-Reyero, 2011). These approaches have provided relevant ecological informations, and their association with high-throughput omics techniques could expand our vision on the organism's responses to toxicants, increase the robustness of the assessment and provide novel evidenced based questions (Villeneuve and Garcia-Reyero, 2011; Gouveia et al., 2019; Vieira et al., 2020). As a result of this intersection, promising novel approaches have become available, such as Ecotoxicoproteomics. It has focused on the investigation of alterations in the proteome of animals through techniques such as mass spectrometry, with the aim to identify (novel) mechanisms of action of environmental contaminants and identification of potential biomarkers (Lemos et al., 2010; Monsinjon and Knigge, 2007; Gouveia et al., 2019). Recently, we have successfully applied the FET test coupled with proteomics analysis to broaden the understanding of the toxic effects of the pesticide 3,4-dichloroaniline on 96 h old-zebrafish larvae (Vieira et al., 2020).

This work aimed to evaluate the toxicity of Cry1C, Cry1F and Cry1Ab on zebrafish embryos and larvae, allying the embryotoxicity test and proteomics analysis to investigate the acute effects of a predicted worst-case scenario concentration of Cry proteins (set to 1.1 mg/L). The choice of this concentration places the proposed experimental approach in a condition closer to what could happen in a very pessimistic scenario of water contamination with Cry proteins. In addition, our previous experience in toxicity tests with other Cry proteins has shown that, in general, these toxins have no or few effects on vertebrates (Farias et al., 2015a, 2015b), which is corroborated by many other studies (Gao et al., 2018; Hammond

et al., 2006; Schrøder et al., 2007; Koch et al., 2015). For this reason, we avoided the use of very low concentrations such as those already determined in some aquatic environments (<130 ng/L) (Tank et al., 2010; Strain and Lydy, 2015), and we set out to start with the worst possible scenario in order to emphasize the safety of these proteins or advise to conduct further studies at lower concentrations.

## 2. Material and methods

### 2.1. Bacillus thuringiensis Cry proteins

Cry1F, Cry1Ab and Cry1C proteins, with a purity around >90%, were obtained commercially from Dr. Marianne Pusztai-Carey, Associate Professor at the School of Medicine, Department of Biochemistry, Case Western Reserve University, and stored at −20 °C until further analyses.

### 2.2. Maintenance and rearing of zebrafish

A wild type strain of zebrafish (*D. rerio*) was reared in the animal facility from the Department of Biology (Federal University of Ceara, Fortaleza, Brazil). The maintenance and obtaining of embryos were performed as described in a previous study (Vieira et al., 2020).

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

### 2.3. Fish embryo acute toxicity (FET) test

The FET test was performed with Cry1F, Cry1Ab and Cry1C according to OECD's guideline number 236 (OECD, 2013), with a modification. This was the use of a single concentration of each Cry protein in the exposures (as a limit test), which corresponded to the worst-case scenario of contamination with Cry proteins in aquatic environments (Carstens et al., 2012). In line with our initial aim and for ethical reasons (using fewer animals), we decided not to test a higher number of concentrations (at least five as recommended). In addition, the limit concentration tested did not cause any adverse effects on the embryos.

For each tested protein, 20 fertilized eggs were placed in 24-well plates (1 embryo per well) and exposed to 1.1 mg/L. Four eggs, exposed to dilution water only, were used as internal plate controls. An additional plate containing embryos only in dilution water (negative control) was also incubated. Eggs with up to 3 hpf (hours post fertilization) of age were exposed to Cry1F, Cry1Ab and Cry1C for 96 h, and embryos were analyzed every 24 h for the apical endpoints: egg coagulation, lack of somite formation, lack of detachment of the tail-bud from the yolk sac and lack of heartbeat. Negative control and Cry proteins solutions were also renewed after 24 h. Observations were performed in a stereo microscope (80× magnification) and photographed (Nikon). After 96 h, the surviving larvae were analyzed and rapidly frozen by immersion in liquid nitrogen and stored at −80 °C for quantification of biomarkers. The suitability of embryos produced by the zebrafish strain used was assessed to the same batch of embryos employed in the test with Cry proteins by exposure to 4.0 mg/L 3,4-dichloroaniline (positive control) as recommended, presenting a mortality rate above 90%.

### 2.4. Biomarker assays (AChE, CAT and GST)

To perform the dosage of enzyme biomarkers, 20 embryos were thawed in ice and homogenized in 0.1 M phosphate buffer, pH 7.4. After 2 h defrosting, samples were centrifuged at 11,500×g, for 15 min, 4 °C. The supernatant was removed and subsequently used for dosage of the following enzymes: acetylcholinesterase (AChE), catalase (CAT) and glutathione-s-transferase (GST). AChE activity was performed according to Domingues et al. (2010), while CAT and GST activities were performed according to Domingues and Gravato (2018). Enzymatic assays were performed using 4 replicates, and the activity of each enzyme was estimated as nanomoles of hydrolysed substrate per minute (each nanomolar being equivalent to one enzyme unit) per mg of protein. Protein concentration in each sample was quantified using the method described by Bradford (1976).

### 2.5. Exposure of embryos to Cry1C, Cry1F and Cry1Ab proteins for proteomic analysis

For proteomic analysis, 20 embryos of zebrafish up to 3 hpf were placed into 24-well plates (1 embryo per well) with each well containing 2 mL Cry solution (1.1 mg/L) or dilution water (negative control). Subsequent steps in this section were all carried out as described by our group in a previous work (Vieira et al., 2020).

### 2.6. Protein extraction, sample preparation for LC-MS/MS, MS data analyses and label-free quantification

All steps of proteomic analysis were performed as described in a previous study published by our research group (Vieira et al., 2020), with only some modifications. Briefly, the protein extraction was performed separately for each experimental group replicate. Control and tests 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. This study used a bottom-up gel free approach. For this purpose, 300 μg proteins were resuspended for LC-MS/MS analysis. The data obtained from MS were analyzed against the reference proteome of *D. rerio* available in UniProt (November 2018), using PatternLab for Proteomics 4.13 (Carvalho et al., 2016, 2012). Finally, for 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 (Zybailov et al., 2006). The TFold module was used to evaluate the levels of differentially expressed proteins (DEPs) (Carvalho et al., 2016). This module is based on a theoretical FDR estimation (Benjamini and Yekutieli, 2001) 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).

### 2.7. Statistical analysis

The data were displayed as mean ± standard deviation (SD) and the means were compared with each other through analysis of variance (ANOVA) followed by Dunnett's test. The results were considered statistically significant only when $p < 0\ 05$.

## 3. Results and discussion

Cry1C, Cry1F and Cry1Ab are proteins used in *Bt* plants, including soy, corn and cotton. Given the reports of the entry of Cry proteins into aquatic environments, it is relevant to evaluate their effects on aquatic organisms. Thus, we sought to investigate the effects of Cry1C, Cry1F and Cry1Ab toxins on zebrafish embryos and larvae using a worst-case concentration of 1.1 mg/L based on the calculations by Carstens et al. (2012). For that, we propose to ally a traditional toxicity approach (i.e. zebrafish embryotoxicity test and

dosage of enzyme biomarkers) to contemporary toxicological assessments through proteomics analysis.

In the FET test conducted with zebrafish embryos, all parameters - hatching rate, water temperature, dissolved oxygen, pH and the survival rate of embryos in the negative control group (100%) - were in accordance to those preconized by OECD (2013). Survival rates of zebrafish larvae after 96 h of exposure, for embryos treated with Cry1C, Cry1F and Cry1Ab proteins (1.1 mg/L), are shown in Fig. 1. The difference in survival between negative control and Cry-treated embryos was not significant ($p < 0.05$). Furthermore, no malformation was observed in larvae after 96 h of exposure. This is in line with previous studies with Cry1C, which also reported no adverse effects on zebrafish embryos and larvae after 132 h of exposure to increasing concentrations of this protein (0.1–10 mg/L) (Gao et al., 2018). In turn, Grisolia and collaborators (2009) showed that at very high concentrations of the Cry1Ab protein (100 and 150 mg/L), the zebrafish larvae had a mortality rate of up to 100%. It is important to note that these tested concentrations are several orders of magnitude higher than those found in the environment, as mentioned earlier. The lack of toxic effects (lethal and sublethal effects) of Cry1C, Cry1F and Cry1Ab to the early stages of the zebrafish life described here is in agreement with several studies that demonstrated the innocuousness of these proteins for a great diversity of vertebrate and invertebrate organisms (Baktavachalam et al., 2015; Romeis et al., 2019).

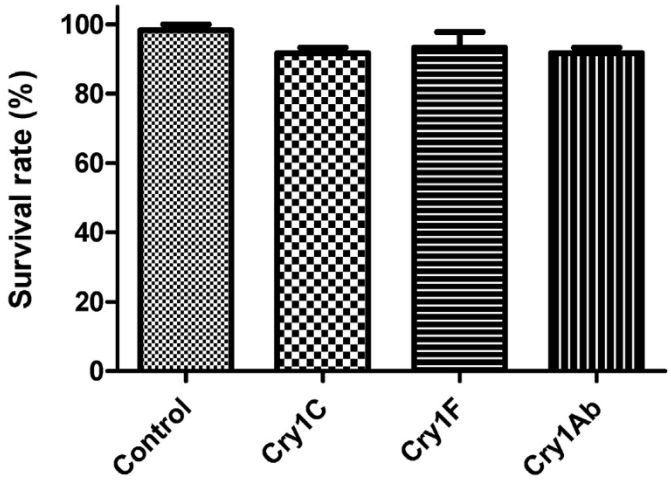


**Fig. 1.** Survival rate of zebrafish embryos exposed to three Cry proteins. Embryos up to 3 hpf were exposed in 24-well plates to Cry1C, Cry1F and Cry1Ab at a concentration of 1.1 mg/L and monitored every 24 h for 96 h. No statistical difference was observed between the treatments and the control group (ANOVA, $p < 0.05$).

Biomarker dosage showed that the activity of enzymes AChE, GST and CAT was not affected in comparison to control (Fig. 2). In an ecotoxicological context, dosing these classical biomarkers is used as a parameter for risk assessment of environmental pollutants such as Cry proteins. It has been shown that AChE, GST and CAT are extremely sensitive to chemical pollutants (Domingues et al., 2010; Domingues and Gravato, 2018); nevertheless, no work in the literature has reported changes in the levels of these enzymes in zebrafish related to exposure to Cry proteins. This is in line with a previous study that also showed that CAT activity is not affected upon exposure to Cry proteins Cry1C e Cry2A (Gao et al., 2018). Proteomics analysis of treated larvae resulted in the identification of 1,178, 1137 and 1265 proteins for Cry1C, Cry1F e Cry1Ab, respectively, using a gel-free/Label-free (using an FDR threshold of 0.01 and considering protein redundancy) approach, as shown in Fig. 3. Quantitative analysis of proteins shared by Cry1C-treated and control groups led to identification of 6 differentially expressed proteins (DEPs). Two DEPs were identified for Cry1F, while Cry1Ab modulated 7 proteins in total (Table 1 and Fig. 3). These results suggest that these toxins do not cause considerable changes in the proteome of zebrafish larvae compared to other stressors. Recent proteomic studies have shown that the pesticides ametrin and dieldrin were able to deregulate a total of 289 and 112 proteins, respectively, in zebrafish larvae (Lin et al., 2018; Simmons et al., 2019), much higher values than those found in this work. Therefore, compared to other compounds, Cry proteins do not extensively alter the proteome of zebrafish. Supplementary material 1, 2 and 3 have a detailed description of treatment-specific proteins.

Among Cry1C DEPs, we detected vitellogenin 2 (Vtg2) and myosin, heavy chain b (Myhb), both upregulated, and type I cytokeratin (Cyt1), down-regulated (Table 1). For Cry1F, two proteins – vitellogenin 1 (Vtg1) and heterogeneous nuclear ribonucleoprotein A/Ba (Hnrnpaba) – were significantly upregulated (Table 1). On the other hand, among Cry1Ab 7 DEPs, we highlight actinb2 (Actb2), ckmb protein (Ckmb) and creatine kinase muscle a (Ckma) proteins, all related to ATP binding, besides viltellogenins 6 and 7 (Vtg6 and Vtg7).

Vitellogenins, cytoskeletal keratins and myosins are among the protein families identified as frequently altered by stressors in a meta-analysis study with 25 protein families in zebrafish. Cytoskeletal keratins occupy the second position, while myosins can be found in the fourth place (Groh and Suter, 2015). In zebrafish, Vtg1,Vtg2, Vtg6 and Vtg7 are among the proteins responsible for transporting lipids, which is essential for larval development (Sullivan and Yilmaz, 2018), while Cyt1 is associated with the cell migration involved in gastrulation (Pei et al., 2007). Moreover, Vtg1,

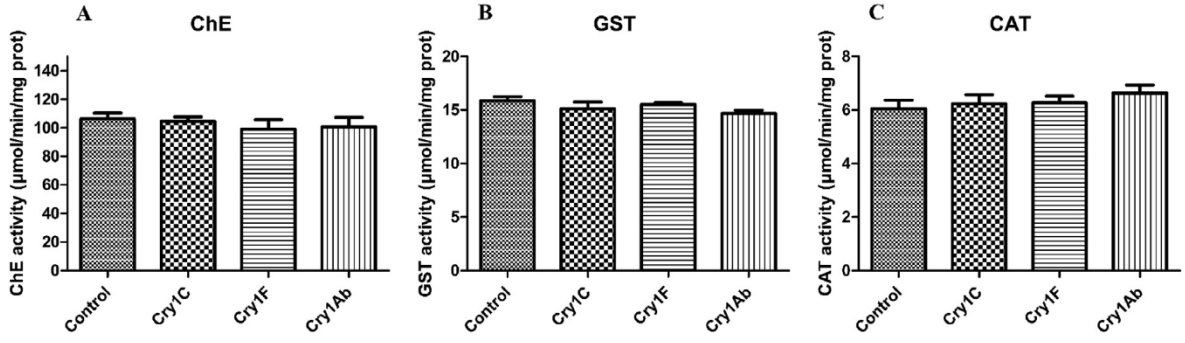


**Fig. 2.** Acetylcholinesterase (ChE), glutathione-s-transferase (GST) and catalase (CAT) activities measured in zebrafish embryos exposed to Cry proteins (1.1 mg/L) for 96 h. Variation of biomarkers activities (µmol/min/mg protein) was expressed as mean value ± standard error (n = 4). A, ChE activity; B, GST activity; and C, CAT activity. No statistical difference was observed between the treatments and the control group (ANOVA and Dunett test as the *post hoc*, $p < 0.05$).

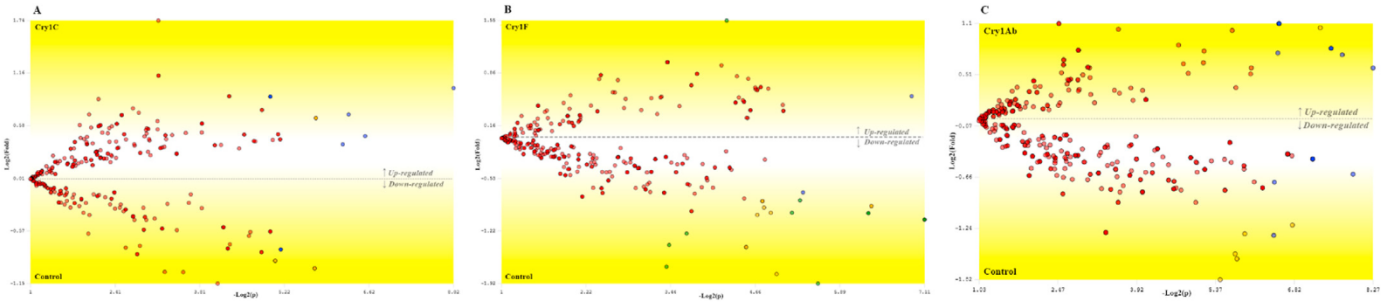


**Fig. 3.** Volcano plots and quantitative analysis of proteins shared by each treatments (-log2 p-value in the x-axis and log2 fold change (FC) in the y-axis) are indicated in A (Cry1C), B (Cry1F) and C (Cry1Ab). Red dots indicate proteins that do not meet the fold change and FDR criteria established 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 cutoff. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

**Table 1**
Differentially expressed proteins (up- and down-regulated) (FDR < 0.01) in larvae exposed to Cry proteins in comparison to negative control (water).

| Uniprot ID | Protein Name | Log2 Fold Change | Function |
|---|---|---|---|
| ***Cry1C treatment*** | | | |
| Q3T7B3 | Vitellogenin 2 | 2.00 | Cellular response to estrogen stimulus; lipid transporter activity; response to estradiol |
| Q802Z4 | Zgc:66,156 protein | 1.39 | Motor activity; ATP binding; actin filament binding |
| F1QVX3 | Myosin, heavy chain b | 1.30 | Motor activity; ATP binding; actin filament binding |
| A7E2L9 | LOC100002040 protein (Fragment) | 1.63 | Motor activity; actin binding |
| Q7ZU48 | Heterogeneous nuclear ribonucleoprotein | 1.87 | RNA binding |
| Q9PWD8 | Type I cytokeratin | −1.71 | Structural molecule activity |
| ***Cry1F treatment*** | | | |
| A7E2Q6 | Vitelogenin 1 | 1.47 | Cellular response to estrogen stimulus; response to xenobiotic stimulus; antioxidant activity |
| F8W446 | Heterogeneous nuclear ribonucleoprotein A/Ba | −1.64 | RNA binding; sequence-specific DNA binding; |
| ***Cry1Ab treatment*** | | | |
| B2GTB1 | Bactin2 protein | 1.50 | ATP binding |
| Q7T306 | Ckmb protein | 2.14 | ATP binding; phosphocreatine biosynthetic process; kinase activity |
| I3ISU3 | Tubulin, alpha 8-like 4 | 1.67 | Microtubule cytoskeleton organization; microtubule-based process; mitotic cell cycle |
| A2BHA3 | Creatine kinase, muscle a | 1.76 | ATP binding; phosphocreatine biosynthetic process; kinase activity |
| A0A2R8RSX9 | Vitellogenin 6 | −1.54 | Lipid transporter activity; cellular response to estrogen stimulus; response to estradiol |
| A0A2R8RUJ6 | Vitellogenin 7 | −1.64 | Lipid transporter activity; cellular response to estrogen stimulus; response to estradiol |
| Q566W6 | Cold-inducible RNA-binding protein a | −2.49 | Positive regulation of mRNA splicing, via spliceosome; RNA binding; positive regulation of translation |

dysregulated in Cry1F-treated larvae, is one of the most common stressor-induced protein in zebrafish (Groh and Suter, 2015).

Overall, DEPs from each experimental group were further analyzed to identify enriched gene ontologies (GO) to obtain insights into the biological functions of these proteins. Three biological processes were found to be enriched for dysregulated proteins of Cry1Ab-treated larvae, which are “phosphocreatine biosynthetic process”, “response to estradiol” and “cellular response to estrogen stimulus”. The main proteins involved in such processes were Ckmb, Ckma, Vtg6 and Vgt7. These data suggest that Cry1Ab, at the concentration of 1.1 mg/mL may disturb phosphocreatine formation.

Although alterations at the phenotypic level were not observed, we were able to identify small changes at the molecular level. Furthermore, the Cry proteins investigated here show that they affect vitellogenins in a global way – even though the underlying mechanisms remain unknown. Besides that, Cry1C e Cry1F deregulated heterogeneous nuclear ribonucleoproteins (Hnrnpa0l and Hnrnpaba, respectively), both implicated in mRNA processing and gene regulation (Chaudhury et al., 2010). However, this small set of altered proteins in the zebrafish larvae treated with Cry proteins is likely to be related to an adaptive response to the presence (in high concentration) of a xenobiotic, given the absence of deaths or any other toxicity endpoint (Siew et al., 2006; Bambino and Chu, 2017).

In addition, it is important to emphasize that we are testing the worst scenario of contamination with these proteins, in which all Cry protein expressed at a certain time in a *Bt* corn farming would be solubilized in a body of water (Carstens et al., 2012). The concentration of the Cry proteins tested in this work is much higher (1 million times bigger) than the maximum concentration of Bt toxins (Cry1Ab) detected in aquatic environments, which is < 130 ng/L (Tank et al., 2010; Strain and Lydy, 2015). Therefore, it is very unlikely that such low concentrations will cause damage to aquatic vertebrates (mainly fishes), considering that even using a high-throughput omics technique, we have not identified any evidence of strong toxicity. Therefore, this study adds to other investigations that describe the innocuousness of these proteins in vertebrates (Dryzga et al., 2007; Cao et al., 2010; Wang et al., 2013; Farias et al., 2015a, 2015b).

## 4. Conclusion

In this work, we evaluated the effects of Cry1C, Cry1F and Cry1Ab *Bt* proteins in zebrafish embryos and larvae considering the worst-case scenario for aquatic environmental contamination (1.1 mg/L). At this concentration, these proteins did not cause adverse effects observable in the zebrafish early life stages, either

by verifying phenotypic endpoints of toxicity or alterations in representative enzymatic biomarkers. At the molecular level, Cry proteins tested lead to very small changes in the proteome of zebrafish larvae. Overall, these data lead to the conclusion that Cry1C, Cry1F and Cry1Ab proteins, even at a very high concentration, have limited effects in the zebrafish early life stages. It is quite reasonable to say that, in a natural setting, these proteins would not have deleterious effects on aquatic vertebrates.

## Credit author statement

Leonardo Vieira, Methodology, Investigation, Visualization, Writing - original draft, Writing - review & editing. Denise Hissa, Conceptualization, Methodology, Investigation, Resources. Terezinha Souza, Writing - original draft, Writing - review & editing. Íris Gonçalves, Writing - original draft. Joseph Evaristo, Investigation. Fábio Nogueira, Resources, Methodology. Ana Carvalho, Resources. Davi Farias, Funding acquisition, Project administration, Conceptualization, Methodology, Resources, Supervision.

## Declaration of competing interest

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

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2020.128538.

## Data availability

Supplementary material 1, 2 and 3.

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