Hepatic proteome and toxic response of early-life stage rainbow trout (*Oncorhynchus mykiss*) to the aquatic herbicide, Reward®

by

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Approval

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Ethics Statement

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Abstract

The objective of this study was to examine the acute toxicity and sub-lethal effects of the commercial formulation of diquat dibromide, Reward® Landscape and Aquatic Herbicide, on multiple early-life stages of rainbow trout exposed to environmentally relevant concentrations. The continuous exposure 96 h LC_{50} derived for juvenile feeding fry aged 85 d post-hatch was 9.8 mg/L. Rainbow trout eyed embryos and juvenile feeding fry were also exposed to concentrations of Reward® ranging from 0.12 to 10 mg/L during two 24 h pulse exposures separated by 14 d of rearing in fresh water to mimic the manufacturers instructions for direct applications to water bodies. Effects on growth and development were evident at 9.25 mg/L during the embryo/alevin exposures, but not in feeding juveniles, indicating a higher sensitivity of the early life stage fish. Quantitative proteomic assessment and subnetwork enrichment analyses were conducted on hepatic proteins for both life stages to evaluate protein expression changes after 0.37 mg/L diquat via Reward® exposure. Unique cellular process expression profiles for pre-feeding swim-up fry and for feeding juvenile fish were observed, reflecting differences between the two life stages in sub-cellular responses after diquat dibromide exposure. Hepatic proteome effects were more dramatic in the pre-feeding swim-up fry with 315 proteins significantly different between the control and fish exposed to Reward®, while in the later life stage feeding fry, only 84 proteins were significantly different after Reward® exposure. This study is the first to report the sub-cellular and whole organism level effects of diquat dibromide in a commercial formulation and demonstrates that numerous changes at the protein level occur at environmentally relevant concentrations based on aquatic application rates.

Keywords: Reward®; Proteomics; Diquat dibromide; Rainbow trout; Sub-network enrichment analysis

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Table of Contents

Approval ... ii
Ethics Statement ... iii
Abstract ... iv
Acknowledgements ... v
Table of Contents ... vi
List of Tables ... viii
List of Figures ... ix
List of Acronyms ... x
Glossary ... xi

Chapter 1. Introduction ... 1
1.1. Pesticides in Canada ... 1
1.2. Reward® Landscape and Aquatic Herbicide ... 2
1.2.1. Chemical Properties and Environmental Fate ... 3
1.2.2. Diquat Toxicity in Plants and Non-Target Animals ... 5
1.3. Rainbow trout (*Oncorhynchus mykiss*) ... 8
1.4. Proteomics as an Ecotoxicological Tool ... 9
1.5. Research Objectives ... 11
1.6. Tables ... 12
1.7. References ... 13

Chapter 2. ... 18
2.1. Methods ... 18
2.1.1. Chemicals ... 18
2.1.2. Animals ... 19
2.1.3. Exposure Apparatus ... 19
2.1.4. In Vivo Rainbow Trout Exposures to Reward® ... 19
Acute lethality 96 h rainbow trout exposure to Reward® ... 19
Pre feeding early-life stage pulse dose exposure to Reward® ... 20
Juvenile feeding life stage pulse dose exposure to Reward® ... 22
2.1.5. Fish Euthanization and Tissue Collection ... 23
2.1.6. Hepatic Tissue Protein Extractions ... 23
2.1.7. Protein Quantification ... 24
2.1.8. Pathway Analysis ... 26
2.1.9. Statistical Analysis ... 26
2.2. Results ... 27
2.2.1. Water quality and pesticide exposures ... 27
2.2.2. Acute Lethality Toxicity Test ... 28
2.2.3. Pre Feeding Early Life Stage Pulse Dose Exposure to Reward® ... 28
Proteomics ... 29
Pathway Analysis ... 30
2.2.4. Juvenile Feeding Life Stage Pulse Dose Exposure to Reward® ... 31

Proteomics 32
Pathway Analysis 33
2.3. Discussion 34
2.3.1. Reward® Effects on Growth, Development and Survival 34
2.3.2. Proteome Response and Underlying Subnetworks after Pulse Dose Reward® Exposure 36
2.4. Conclusion and Future Work 41
2.5. Tables 43
2.6. Figures 50
2.7. References 66

Appendix A. Supplemental Information 69

List of Tables

Table 1.1 Application Rates for Reward® Landscape and Aquatic Herbicide according to label instructions 12
Table 1.2 Chemical properties and structure of of diquat dibromide 12
Table 2.1 Summary of Rainbow Trout Acute Toxicity Tests (96 h LC_{50}) to Reward® Landscape and Aquatic Herbicide 43
Table 2.2 Summary of Rainbow Trout Pulse Exposures to Reward® Landscape and Aquatic Herbicide 43
Table 2.3 Nominal and measured concentrations of diquat ion during acute and sub-chronic rainbow trout exposures to Reward®. 44
Table 2.4 Early life stage rainbow trout sub-network enrichment analysis (SNEA) for cell process in livers collected from water controls versus Reward® (0.37 mg/L diquat ion) exposed fish 45
Table 2.5 Juvenile life stage rainbow trout sub-network enrichment analysis (SNEA) for cell process in livers collected from water controls versus Reward® (0.37 mg/L diquat ion) exposed fish 48

List of Figures

Figure 2.1 Rainbow Trout exposure apparatus set-up...50

Figure 2.2 Concentration response curve for the continuous 96 h LC_{50} toxicity test exposure to Reward® Landscape and Aquatic Herbicide........................51

Figure 2.3 The effects of rainbow trout following two 24 h pulse exposures to Reward® 14 d apart on survival 52

Figure 2.4 The effects of early life stage rainbow trout (embryo through to swim-up fry) following two 24 h pulse exposures to Reward® 14 d apart on length and weight...53

Figure 2.5 Percent distribution of Gene Ontology (GO) annotations of biological process and functions for identified hepatic proteins with significant expression changes from treatment fish vs controls for early life stage rainbow trout (embryo to swim-up fry) 54

Figure 2.6 Variations in the expression of hepatic proteins after early-life stage rainbow trout exposure to diquat dibromide using Reward® formulation. 55

Figure 2.7 Pathway studio analysis for protein network for differentially expressed proteins with the process of sterol regulatory element binding protein....56

Figure 2.8 Pathway studio sub-network enrichment analysis for differentially expressed proteins with the process of splice site selection 57

Figure 2.9 Pathway studio sub-network enrichment analysis for differentially expressed proteins with the process of mRNA metabolism and protein splicing. 58

Figure 2.10. Pathway studio sub-network enrichment analysis for differentially expressed proteins with the master gene regulator CASP3....................59

Figure 2.11 The effects to juvenile life stage rainbow trout (66-86 d post hatch) following two 24 h pulse dose exposure to Reward® 14 d apart on length and weight...60

Figure 2.12 Percent distribution of Gene Ontology (GO) annotations of biological process and functions for identified hepatic proteins with significant expression changes from treatment fish vs controls for juvenile life stage rainbow trout (66-86 d post hatch)...61

Figure 2.13 Variations in the expression of hepatic proteins after juvenile rainbow trout exposure to diquat dibromide using Reward® formulation...............62

Figure 2.14 Pathway studio sub-network enrichment analysis for differentially expressed proteins with the process of Atk/mTOR 63

Figure 2.15. Pathway studio sub-network enrichment analysis for differentially expressed proteins with the process of glycogen degradation................64

Figure 2.16. Pathway studio sub-network enrichment analysis for differentially expressed proteins with the process of ribosome biogenesis and assembly and regulation of translational fidelity......................................65

List of Acronyms

AOP	Adverse outcome pathway
BC	British Columbia
BCF	Bioconcentration Factor
DFO	Fisheries and Oceans Canada
HRAC	Herbicide Resistance Action Committee
IRIS	International Agency for Research on Cancer
iTRAQ	Isobaric tagging for relative and absolute quantitation
Kow	Log Octanol-water coefficient
LAC	Library and Archives Canada
LC50	Lethal Concentration to 50 % of organisms
LOEL	lowest observable effect level
NADPH	nicotinamide adenine dinucleotide phosphate
NCAG	National Contaminants Advisory Group
NOEL	Non-observable effects level
PMRA	Pesticide Management Regulatory Agency
ROS	Reactive Oxygen Species
SFU	Simon Fraser University

Glossary

Acute Toxicity	describes the adverse effects of a substance that result either from a single exposure or from multiple exposures in a short period of time
Adverse Outcome Pathway	defines a sequence of key events commencing with the interaction of a stressor (e.g., toxicant) on target cell or tissue and resulting in an adverse outcome for an organism
Alevin	fish life stage after hatch with yolk sac still present
Chronic Toxicity	describes the adverse effects as the result of long term exposure to a toxicant or other stressor.
Downregulated	the process by which a cell decreases the quantity of a cellular component such as RNA or protein in response to an external stimulus
Ecotoxicology	the branch of science that deals with the nature, effects, and interactions of substances that are harmful to organisms living in the environment.
Gene	a distinct sequence of nucleotides forming part of a chromosome, the order of which determines the order of monomers in a polypeptide or nucleic acid molecule which a cell may synthesize
Health Stack	vertically-stacked incubation trays used to rear eggs and alevins
in vitro	performed or taking place in a test tube, culture dish, or elsewhere outside a living organism
in vivo	performed or taking place in a living organism
Pesticide	A product that is manufactured and sold for means to directly or indirectly control, mitigate or destroy any pest
Photosystem II	protein complex found in the thylakoid membrane of plants used in photosynthesis
Proteome	the entire complement of proteins that is or can be expressed by a cell, tissue, or organism
Proteomics	the study of proteomes and their function
Redd	series of depressions dug into gravel substrate by a female salmonid in which eggs are deposited
Salmonid	A fish of the salmon family (Salmonidae
Swim-up	early-life event where by alevins swim-up through gravel into the water column for feeding, typically when yolk sac is nearly depleted.

Thesis	an extended research paper that is part of the final exam process for a graduate degree. The document may also be classified as a project or collection of extended essays.
Upregulated	the process by which a cell increases the quantity of a cellular component such as RNA or protein in response to an external stimulus

Chapter 1. Introduction

1.1. Pesticides in Canada

A pesticide is defined as a product that is manufactured and sold for means to directly or indirectly control, mitigate or destroy any pest (Health Canada, 2017). Pesticides are formulated for their associated target pest (e.g., plants or insects) and are commonly classified according to target pest including herbicides, insecticides, fungicides or vertebrate pest toxicants (British Columbia Ministry of Environment, 2010; Health Canada, 2017). Herbicides are the most prevalent pesticide category used globally with the largest proportion used in the agricultural industry, but herbicides are also routinely used in the forestry sector and for a variety of other purposes to control plant pests (i.e., rights of way, industrial and urban areas; Solomon, Dalhoff, Volz, & Van Der Kraak, 2013). In Canada, herbicides are regulated by the Pest Management Regulatory Agency (PMRA) within Health Canada, as part of the *Pest Control Products Act* of Canada. The PRMA is responsible for the registration of new pesticide products, the re-evaluation of existing registered pesticide products, and determining food residue limits of pesticides in Canada (Environment and Climate Change Canada, 2011). The National Pesticides Monitoring and Surveillance Network administered by Environment and Climate Change Canada (ECCC) was developed to evaluate and report on pesticide levels and their transformation products to protect freshwater systems in Canada and to support the PMRA mandate (National Pesticides Monitoring and Surveillance Network, 2018).

Currently there are over 7,000 registered pesticides in Canada with an estimated total of 90 million kilograms sold in Canada in 2011 (Health Canada, 2011). Of the total sales in Canada, 59% was estimated to be chemicals used as herbicide, and is attributed to the wide usage in the agricultural sector (Health Canada, 2011). The National Contaminants Advisory Group (NCAG), within Fisheries and Oceans Canada (DFO), is responsible for providing and facilitating scientific research on the biological effects of contaminants on aquatic organisms. Particularity, NCAG is responsible for conducting research on priority contaminant issues for the DFO, which include the assessment of pesticides that require additional biological information for risk assessments or registration purposes (NCAG, 2018).

One method of anthropogenic ecosystem management of aquatic and wetland plants is controlling overgrowth of non-native species or plants involved in eutrophication that may change or limit the productivity of aquatic systems (Simsiman and Chesters, 1975). Aquatic herbicides are a group of pesticides used to control aquatic plants in surface waters but are poorly studied with respect to their adverse effects on non-target aquatic wildlife compared to agricultural use pesticides. Biological effects and toxicity to non-target organisms, such as fish, following application of aquatic herbicides is typically considered in risk management, and pesticide registration decisions (ECCC, 2011).

1.2. Reward® Landscape and Aquatic Herbicide

Reward® Landscape and Aquatic herbicide, herein referred to as "Reward®", is registered in Canada (registration number 26271) for restricted use and requires applicators to obtain use permits that outline strict conditions for application (Health Canada, 2017). As part of the PMRA role, registered products are re-evaluated on a 15-year cycle to allow for new product information or related scientific advances to be incorporated into pesticide renewal considerations (PMRA, 2018). Reward® will be re-evaluated for renewal after December 31, 2020 (Health Canada, 2018). Reward® is currently not monitored under the National Pesticides Monitoring and Surveillance Network program, therefore current information on environmental levels in Canada are unknown (NCAG, 2018).

Reward® contains the active ingredient diquat dibromide and is registered for use on submersed or floating weeds in aquatic environments and as a terrestrial herbicide in nurseries, commercial greenhouses, seed crops, and for landscape purposes on industrial, commercial, recreational and residential lands (Syngenta, 2005). In Canada diquat dibromide is also the active chemical in other terrestrial herbicides (e.g., Syngenta Reglone Ion, Syngenta Desica, Sharda Diquat 240) used for food crops (e.g., beans potatoes and oats) and for non-cropland weed control that can be applied to a site by ground or aerial spray equipment (Health Canada, 2010). These terrestrial applications can also result in contamination of surface or ground water via leaching into soils and/or runoff into nearby aquatic receiving environments during precipitation events or enter the water directly through spray drift during applications (Gandar et al, 2017; Boithias et al, 2011). The Reward® application instructions for submersed weeds specifies the application should be conducted during active growth periods and when water

temperatures exceed 10°C (i.e., typically during spring and summer months in Canada; Syngenta 2005). To mitigate effects to aquatic organisms, some key application restrictions are included on the Canadian Reward® label. These application restrictions include: 1) for treatment of dense weeds, it is recommended to treat only 30 to 50 % of the water body at one time as decomposition of dead plant material may result in a loss of oxygenated waters and cause adverse effects to fish; 2) a 14 day period is required between multiple treatments; and, 3) no applications are permitted into aquatic systems where commercial fisheries take place that produce fish meal or concentrated fish protein products (Health Canada, 2008).

Application rates for Reward® are dependent on the density of growth of the target weeds and the depth and size of the water body (Syngenta, 2005). For light plant growth areas, the target concentration is 0.18 mg/L, and for dense growth areas the target concentration is 0.37 mg/L. Typical application rates are shown in Table 1.1 (Syngenta, 2005). Different application methods may be employed depending on plant type, such as submersed or floating weeds (Syngenta, 2005). For submersed weeds (e.g., Hydrilla, Bladderwort, Coontail) Reward® can be injected below the water surface using a boat mounted injection tube (Syngenta, 2005). The application can be completed along transect lines over regular intervals to ensure even application (Syngenta, 2005). For surface plants, Reward® can be applied directly into the water from shore or can be aerial applied or sprayed over the surface of the water body. Adhering to approved application rates thus depends on accurately identifying depth and size of the water body and the density of plant growth.

1.2.1. Chemical Properties and Environmental Fate

Reward® contains 373 g/L of the active chemical diquat dibromide [6,7-dihydrodipyriod (1,2-a:2',1'-c) pyrazinediium dibromide], and has a molecular weight of 344 g/mol. Diquat dibromide (CAS 85-00-7) is a quaternary ammonium compound that once applied to water dissociates into a divalent cation (molecular weight 221 g/mol). The diquat cation, herein referred to as "diquat", migrates into plant tissue and behaves as a fast-acting desiccant (Syngenta, 2016). Chemical properties for diquat dibromide are summarized in Table 1.2. Diquat dibromide has a high solubility in water making it ideal for use as an aquatic herbicide. The log octanol-water coefficient (Kow) is low and reported to be -4.6 suggesting diquat does not partition into the octanol or lipid phase readily,

indicating it has a higher affinity for water and is not likely to partition into biota and therefore is not likely to bioconcentrate in biota or bioaccumulate in the food chain (Ritter et al, 2000; Siemering et al, 2008; Emmett, 2002; Chiovarou and Siewicki, 2007). The high soil adsorption coefficient (kd) for silt and clay (10,000 to 60,000) suggests the chemical binds strongly to sediment or soil and is immobile in this media (Chiovarou and Siewicki, 2008). Tucker et al. (1967) reported that the diquat binding affinity for soils is dependent on the composition of the soil, where generally diquat will bind more strongly to clay soils. This study showed diquat tightly bound in clay soil could only be removed by denaturing the soil following reflux with sulfuric acid, while loosely bound diquat could be removed with aluminum ions (cation exchange) and unbound diquat in soils could be removed through water leaching. Tucker et al. (1967) also showed that soils containing a higher composition of organic material leached diquat more readily through water elution. Although a few studies have been conducted on the adsorption capacity of diquat to sediment and soils, little is known about the capacity of sediment adsorption after repeat exposures and chronic effects and bioavailability to sediment dwelling organisms such as benthic invertebrates. In addition, depending on the *in situ* soil characteristics diquat may runoff from terrestrial agricultural use applications into aquatic receiving environments since diquat affinity for soil can vary based on composition.

Dissipation of diquat from the water column is thought to be rapid and typically occurs within the first 24 h following application due to the rapid uptake into plant tissue and adsorption to sediment and suspended particles (Ritter et al, 2000; Grzenda et al, 1966; Emmett, 2002; Syngenta 2015). For sediments containing high amounts of clay, diquat is considered to strongly bind to sediment, and therefore would be less bioavailable to aquatic biota (Ritter, 2000). Microbial degradation in soil and sediment is slow and it is estimated that only 5-10 % is degraded per year (Emmett, 2002). Based on the low log Kow value for diquat, the bioconcentration potential or uptake from water into biota is anticipated to be low, and this is supported by the bioconcentration factor (BCF) reported in literature. The reported bioconcentration factor for bluegill sunfish and tilapia was found to be ~ <1.0, suggesting that diquat is not likely to bioconcentrate in fish (Emmett, 2002). Additionally, with the predicted rapid dissipation rates from the water column and binding to sediment, diquat is not anticipated to be bioavailable to fish for long exposure durations *in situ*. However, based on the application instructions for the use of Reward® for aquatic plants in water bodies, exposure would be associated with multiple pesticide application

events resulting in multiple short-term exposures or pulse exposures. Currently no data is available in literature on acute pulse exposure of diquat to fish or other aquatic organisms and the adverse effects of diquat on sediment dwelling organisms (i.e., benthic invertebrates) has not been clearly determined (Emmett, 2002; Bouetard et al, 2013).

1.2.2. Diquat Toxicity in Plants and Non-Target Animals

The toxic mode of action for herbicides is designed to target plant systems, for example, herbicides can interfere with photosynthetic systems that are absent in animals. Due to this targeted mode of action, herbicides are often considered less toxic to animals particularly under acute exposure conditions; however, the adverse effects of chronic exposures at low levels are not well studied for many herbicides (Solomon et al, 2013). Diquat belongs to the bipyridylium herbicide family, along with paraquat, and is classified as a Group D herbicide according to the Herbicide Resistance Action Committee (HRAC) which groups herbicides based on mode of action (HRAC, 2017). Group D herbicide mode of action in plants involves inhibition of photosystem I which is involved in photosynthesis (Solomon et al, 2013). As a photosystem I inhibitor, diquat ions accept electrons from photosystem I ultimately forming hydroxyl radicals that destroy lipid containing compounds including components of cell membrane, fatty acids and chlorophyll (Dodge, 1982). Lipid peroxidation causes destruction of cell membranes resulting in cytoplasm leakage from cells and ultimately leaf wilting and rapid desiccation (Dodge, 1982). Reward® is a non-selective contact herbicide meaning it will affect only the parts of the plant that the herbicide contacts (Syngenta, 2015).

Based on mammalian toxicity studies diquat dibromide is considered moderately irritating for acute dermal toxicity and is considered slightly toxic for acute oral and inhalation toxicity (US EPA, 1995). Due to a lack of acceptable mammalian studies diquat is not assessed for carcinogenicity under the International Agency for Research on Cancer (IARC) or through the Integrated Risk Information System (IRIS), however in the US EPA risk assessment diquat dibromide is classified as a Group E carcinogen, suggesting evidence of non-carcinogenicity for humans (US EPA, 1995). In a chronic feeding study using Sprague-Dawley rats, fed 5 to 375 mg/L diquat ion by oral gavage over 104 weeks, lens opacity and cataracts were observed. Based on this study the no-observable effects level (NOEL) for rats was determined to be 15 mg/L diquat ion and the lowest observable effect level (LOEL) was determined to be 75 mg/L diquat ion, both based on lens opacity

of the eye. Oxidative stress and hepatic narcosis has been demonstrated in previous toxicity studies conducted on mammalian models both *in vitro* and *in vivo* (Smith et al, 1985; Jones et al, 1981; Akerboom et al, 1982; Rubin and Farber, 1984). This mode of action has been attributed to the redox cycling capability of diquat ion which causes the formation of reactive oxygen species (ROS) such as hydrogen peroxide and the hydroxyl radical (Jones and Vale, 2000; Sandy et al, 1987). Redox cycling of diquat is hypothesized to occur by reaction with cytochrome P450 and in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) (Sandy et al, 1987). Under normal circumstances the body has natural defences to overcome oxidative stress, such as enzyme systems including glutathione peroxidase and catalase; however, when the system is overwhelmed or depleted in response to toxic exposure, oxidative stress can result in cellular membrane damage and ultimately cell death (Hinton et al, 2008). Previous acute toxicity studies conducted on Spargue-Dawley rats exposed via the diet to 20 mg/kg diquat dibromide indicated that glutathione levels are reduced in the liver (Reif et al, 1988) along with NADPH levels (Rawlings et al, 1994). Higuchi et al, (2011) also indicated that diquat ion (0.1 mmol/kg by injection) induced oxidative stress in the liver of male Fisher-344 rats (10 weeks old), and in this case it was related to the release of iron from hepatic ferritin suggesting that transition metal ions may have an important role in the hepatotoxicity of diquat. Although the diquat ion has been implicated in oxidative stress in mammalian models, it has not yet been determined to be the lethal mode of action, particularly after acute exposure.

For aquatic vertebrates such as fish, the water concentrations that are acutely toxic occur in the low mg/L range but chronic environmentally relevant exposure scenario effects are poorly understood. Several aquatic acute toxicity studies were incorporated in the United States Environmental Protection Agency (US EPA) *Registration Eligibility Decision for Diquat Dibromide,* and the US EPA concluded that diquat is slightly to moderately toxic to fish (US EPA, 1995). Studies included in the US EPA assessment to evaluate diquat dibromide toxicity to fish reported adult rainbow trout 96 h LC_{50} values between 14.8 mg/L and <18.7 mg/L. Pimentel et al. (1971) determined the adult 24 and 48 h LC_{50} for rainbow trout to be 90 mg/L and 12.3 mg/L respectively, while the adult fathead minnow 24 h LC_{50} is reported to be 24 mg/L. Adult Chinook salmon were less sensitive with a 48 h LC_{50} of 28.5 mg/L, while northern pike (96 h LC_{50} 16 mg/L) exhibited similar acute toxicity responses to those reported for rainbow trout (Pimentel et al, 1971).

A risk assessment completed by the *Washington State Department of Ecology* on diquat bromide (2002) reported juvenile salmonids were slightly more sensitive to diquat dibromide than adult fish. This risk assessment reported a rainbow trout fingerling (no age/size reported) 96 h LC_{50} of 9.5 mg/L (hardness not specified), 15 mg/L (hardness <50 mg/L), and 14.9 mg/L (hardness >50 to 150 mg/L). In addition, this risk assessment reported a 96 h LC_{50} of 16 mg/L for rainbow trout fingerlings (~ 50 mm length) at a moderate harness (>50 to 150 mg/L). Campbell et al. (2000) reported a 96 h LC_{50} of 14.8 mg/L for juvenile rainbow trout 35 mm in length (0.56 g) and another 96 h LC_{50} of 26 mg/L for rainbow trout 30 mm in length (0.44 g), however no water hardness values or age of animals were included for these studies. In summary the previous studies to date report 96 h LC_{50} values ranging from approximately 9.5 to 25 mg/L for rainbow trout however information on fish age, water quality, and exposure design are not always included. Additional acute studies capturing potential developmental stage specific sensitivities and toxicity modifying factors in fish are necessary to more thoroughly understand the acute toxicity of diquat ion in these non-target organisms.

The chronic toxicity of diquat to aquatic vertebrates has not been extensively evaluated and is therefore poorly understood. A chronic toxicity study conducted by Tapp and Caunter (1986) on juvenile rainbow trout exposed to diquat for 21 d (48.6 mm fingerling) reported an LC_{50} of 2.9 mg/L. Another 34-day chronic toxicity study conducted on early life stage fathead minnow (egg to fry stage) reported a NOEC of 0.12 mg/L and a LOEC of 0.32 mg/L based on growth (Supernant, 1987). Some chronic and acute studies have been conducted on diquat to evaluate oxidative stress as a mode of toxic action. One study conducted by Sanchez et al. (2006) on adult three-spined stickleback examined the chronic effects (21 day exposure) of diquat in relation to known biomarkers for oxidative stress and metabolism. This study suggested that diquat had no effect on hepatic oxidative stress, however chronic exposure inhibited 7-ethoxyresorufine-O-deethylase (EROD) activity and induced glutathione-S-transferase (GST) activity. An acute study conducted by Bouetard et al. 2013 on freshwater snail (*Lymnaea stagnalis*) reported that following acute exposure (5 hours) ROS were increased in hepatocytes suggesting an early oxidative stress response. This study also indicated that diquat may affect other cellular functions related to transcription (Bouetard et al, 2013). Overall, the toxic mode of action of diquat on rainbow trout and other freshwater aquatic species has not been extensively studied. In particular, toxicokinetics and the sub-lethal adverse

effects at low-level environmentally relevant exposure concentrations for diquat are not well studied.

Concentrations of diquat in water are predicted to decrease within days following application since the diquat ion is retained in plant material and/or binds strongly to sediment particles (Siemering et al, 2008). In fish, diquat ion is not anticipated to be readily taken up by gill epithelia or skin due to the cationic chemical properties or to bioconcentrate based on its low log Kow (Schultz et al, 1995; Siemering et al, 2008). Previous studies in rats suggest the ion is also poorly absorbed via the gastrointestinal tract (Daniel and Gage, 1996) and Scott and Corrigan (1990) reported that diquat ion was poorly absorbed by human skin. In a study conducted by Schultz et al, (1995) on bioconcentration of diquat ion in channel catfish, the kidney, liver and bile had the highest residual concentrations, while lower concentrations were found in muscle tissue and gills. Biotransformation of diquat was indicated in the liver and bile as unidentified metabolites (Schultz et al, 1995). This study also showed that diquat was readily eliminated by catfish following an intraperitoneally administered dose, with 64 % of the dose excreted by kidney after 5 h (Schultz et al, 1995). In channel catfish biotransformation of diquat is thought to occur in the liver and bile while the renal pathway is thought to be the main excretion pathway (Schultz et al, 1995). Bioconcentration studies in other fish are necessary to verify the low bioconcentration and excretion of diquat dibromide and any of its metabolites *in vivo* in this taxa.

1.3. Rainbow trout (*Oncorhynchus mykiss*)

Rainbow trout (*Oncorhynchus mykiss*) are a native cold-water salmonid fish species that inhabit freshwater systems from Alaska, through large parts of BC and the Athabasca drainage and down to northwest Mexico. Rainbow trout have been widely introduced beyond their natural range and red-band species can be found across the world (ENV, 2018). Rainbow trout are a popular game fish and are produced by hatcheries for stock in many lakes and ponds across BC (Mellina et al, 2005). Rainbow trout spawn in shallow water during spring or fall laying their eggs in gravel in depressions females create by digging, called redds (ENV, 2018). Eggs hatch into alevins after 4 to 7 weeks, depending on water temperature, and fry emerge from the gravel in the summer months (BC MOE, 2017). As application windows for aquatic herbicides typically coincide with early-life stage development of fish, sensitive life stages can be susceptible to direct

applications of herbicides to water bodies and/or via run-off from terrestrial herbicide applications.

Rainbow trout are a model species for laboratory toxicological studies as eggs are often available year-round from hatcheries. Toxicity data for this model species is prevalent, and many jurisdictions use this as a test species in standardized toxicity testing regimes for a variety of chemical and effluent toxicity testing. For example, in Canada, standardized 96 h acute lethality toxicity tests using rainbow trout must be completed in-order to register pesticides and for monitoring the toxicity of sewage and pulp mill effluent. (ECCC, 2007). Although rainbow trout are a model species for classical toxicology and several studies are available examining the more recent molecular biomarkers endpoints, genomics studies remain challenging due to the lack of a fully sequenced genome for this species. Currently, fully annotated genes and proteins are not available for rainbow trout however, zebrafish and pufferfish genomes have been fully sequenced and work is being conducted on other fish species to improve the teleost database (Snape et al, 2004).

1.4. Proteomics as an Ecotoxicological Tool

In multicellular organisms, signal transduction is a self-regulating biochemical process that responds to a variety of external and internal stimuli (Snape et al, 2004). The stimuli can be in the form of hormones in the body, environmental changes (e.g., changes to external light or temperature), or response to toxicant exposure (Xiong, 1993). Proteins serve numerous cellular functions within an organism and are typically the mediators of cellular responses to stimuli or cellular injury (Xiong, 1993). Signal transduction via proteins in response to toxicant exposure can be an adaptive process that is dependent on the concentration and duration of dose (Snape et al, 2004). Genomic or proteomic changes may be a short-term or long-term toxicological response that, if not corrected, can negatively affect individual fitness (Xiong, 1993). At high doses of toxicant exposure, the signal transduction system may not function properly, and irreversible cellular injury and toxicity may occur (Xiong, 1993). Measuring proteomic responses in an organism can be used to detect potential mechanism of cellular injury and aid in the determination of adverse outcome pathways (AOP) (Rudneva, 2014). The AOP defines a sequence of key events commencing with the interaction of a stressor (e.g., toxicant) on a target cell or tissue and result in an adverse outcome for an organism (OECD, 2017). The AOP defines the effects to the organism at various levels including molecular interactions, cellular

response, organ response, whole organism response and finally population effects (OECD, 2017). Proteomic studies provide insight into initial cellular responses following the exposure of an organism to a toxicant, and therefore aid in development and understanding of the toxic mode of action which help define an AOP. The application of proteomics methods in aquatic ecotoxicology has exponentially grown in the past few years and is an important tool in discovery of sub-lethal and cellular effects in ecotoxicology studies.

There are many different widely used proteomic techniques, with continual advancement in high-throughput methods. Two-dimensional gel electrophoresis (2-DE) has been a routine protein separation technique used in studies since its development in the 1960s. This technique involves linear protein separation by isoelectric point followed by perpendicular separation according to molecular mass using sodium dodecyl sulfate (Martyniuk et al, 2009). This technique may be used less frequently than mass spectrometry-based approaches in recent years due to the fact that it is labour-intensive, has low throughput and poor reproducibility (Hack, 2004). Despite some drawbacks, studies in ecotoxicology still gain valuable information from such an approach. Technological advances that are considered high through-put techniques for protein quantitation have been developed using mass spectrometer (MS) instrumentation and include isotope coded affinity tags (ICAT), stable isotope labeling with amino acids in cell culture (SILAC) and isobaric tagging for relative and absolute quantitation (iTRAQ®). The ICAT technique involves labelling the cysteine thiol groups of proteins using iodoacetamide reagent, thus the technique is limited to identifying proteins with cysteine groups. The SILAC technique also employs isotope labelling with amino acids to cells grown in cell cultures over multiple generations (Martyniuk et al, 2009). Isobaric tagging for relative and absolute quantitation (iTRAQ®) is a common technology currently being used in aquatic ecotoxicology applications to study changes in the proteome (Ross et al, 2004). The iTRAQ® technique employs liquid chromoatography and mass spectrometry (LCMS) instrumentation, often in tandem (i.e., MS/MS) to label the proteins by mass specific tags, fraction by mass, and quantify (Washburn, 2011). In this technique proteins are solubilized and subjected to proteolysis to produce short peptides about 10–20 amino acids in length (Washburn, 2011). Peptides are tagged for identification purposes and spectra for each peptide are run against a database (Edwards, 2011). One advantage of the iTRAQ® technique is that samples from different sources (i.e., control samples and

biological replicates) can be pooled and run in a single experiment, reducing the variability introduced from instrumentation from sample preparation and reducing run costs.

Proteomic assessment allows for evaluation of differentially expressed proteins which can be used in toxicological applications such as examining expression changes between chemically exposed animals and control animals. Sub-network enrichment analysis (SNEA) is a complementary tool often used with proteomics data that applies functional annotation and association of proteins with certain biological processes and pathways. The benefit of pathway analysis is to identify potential enriched biological pathways from large omic datasets that would often be tedious to manually evaluate. Software programs, such as Pathway Studio, allow for input of quantitative proteomics data and use an algorithm and known literature inputs to identify statistically significant enriched pathways. Some limitations of this approach include a lack of ability for tissue specific protein association and predicted pathways are built from mammalian models using existing literature. Despite some limitations, quantitative proteomics has become a widely used and important approach to biomarker discovery along with advances in genomics in order to understand toxicant impacts on a variety of organisms (Han et al, 2017). Quantitative proteomics provide a variety of proteins or genes of interest that can be validated through further detailed bioinformatic or functional genomics studies. Bioinformatic approaches such as pathway analysis will continue to be important in providing functional insight into proteomic datasets as they offer analysis and evaluation of complex protein interactions (Washburn, 2011).

1.5. Research Objectives

The objective of this study was to examine the acute toxicity and sub-lethal effects of environmentally relevant concentrations of a commercial formulation of an aquatic herbicide, Reward®, on multiple life stages of rainbow trout. This thesis describes three main experiments examining the adverse effects of Reward® on rainbow trout. The first experiment conducted investigated the acute toxicity of diquat on the commercial formulation, Reward®, in continuous, 96 h acute toxicity experiments using juvenile rainbow trout (~2-3 months old). The next two experiments were pulse exposures to Reward® using early-life and juvenile developmental stages of rainbow trout. The exposures were two 24 h long pulses of Reward® separated by 14 d of rearing in clean water. Pulse exposures were designed to mimic the application rates of Reward® when

used as an aquatic herbicide, whereby according to the manufacturer's instructions repeat herbicide applications are restricted to every 14 d. The 24 h exposure duration was based on the expected time diquat remains in the water column before dissipation (Syngenta 2015). Several endpoints were measured to examine lethal and sub-lethal effects of Reward® on multiple life stages of rainbow trout. In all experiments, the endpoints measured included survival, morphometrics (i.e., length and weight) and deformity assessment for each fish. For the early-life and juvenile pulse dose experiments, whole fish livers were collected, and proteins were extracted for quantitative proteomics using iTRAQ® techniques. Proteomics data including expression changes (i.e., fold change) were used to conduct a pathway analysis to evaluate changes in cell processes and expression targets. The data obtained from these experiments will be used to address data gaps on toxicological information for early life stage salmonids and will be used in risk assessment and regulatory decision related to pesticide registration.

1.6. Tables

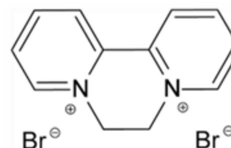
Table 1.1 Application Rates for Reward® Landscape and Aquatic Herbicide according to label instructions

	Gallons of Reward® per surface acre and average water depth				
	1 Foot	**2 Feet**	**3 Feet**	**4 Feet**	**Final Conc.**
1 gal/acre	0.25	0.50	0.75	1.0	0.18 mg/L
2 gal/acre	0.50	1.0	1.5	2.0	0.37 mg/L

The 1gal/acre rate is for light plant growth with a target concentration of 0.18 mg/L and the 2gal/acre rate is to control heavy plant growth with a target concentration of 0.37 mg/L. (Syngenta Crop Protection Inc., 2005).

Table 1.2 Chemical properties and structure of of diquat dibromide

Property	
Molecular Weight[a]	344 g/mol
Vapour Pressure[b]	< 4 x 10^{-9}
Solubility[a]	708,000 mg/L
Log Kow[c]	-4.6 @ 20°C
Half-life water[b]	0.75 d
Half-life sediment[b]	1000 d
Adsorption Coefficient (kd) silt and clay[b]	10,000 to 60,000
BCF[b whole body bluegill sunfish and Talapia]	~ <1.0



[a] Chiovarou and Siewicki, 2007; [b] Emmett, 2002; [c] Ritter, 2000

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Chapter 2.

2.1. Methods

Three main experiments examining the adverse effects of Reward® on rainbow trout were conducted. The first was acute lethality toxicity testing on juvenile rainbow trout to determine the lethal concentration of Reward® causing 50 % mortality of fish over a 96 h exposure (96 h LC_{50}). This was followed by two pulse dose exposures to Reward® that entailed two 24 h pulse exposures separated by 14 d in clean water. Specifically, the second experiment was initiated at the eyed embryo stage and continued over a 26 day period through to the swim-up fry developmental stage (i.e., pre feeding early-life stage exposure). The third experiment was conducted over 18 d on feeding juvenile rainbow trout (i.e., feeding juvenile stage exposure). Each exposure included five geometric series test concentrations, controls and four replicates. During exposures water quality (temperature, conductivity, dissolved oxygen and ammonia) was monitored daily. Survival and fish health observations were made daily and tanks were siphoned daily to remove debris (i.e., food and wastes). Rainbow trout were allowed to acclimatize for 5 d prior to testing. Additional details of each exposure is described below. All exposures were conducted at Alcan Aquatic Center located at Simon Fraser University (SFU), BC, Canada.

2.1.1. Chemicals

The commercial formulation of Reward® was obtained from Syngenta Canada and contains 373 g/L diquat dibromide [6,7-dihydrodipyriod (1,2-a:2',1'-c) pyrazinediium dibromide]. Diquat dibromide is manufactured as a bromide salt but can also be expressed as diquat ion; Reward® contains a concentration of 240 g/L diquat ion. Exposure concentrations were made-up in clean 20 L food grade plastic buckets and diluted with dechlorinated municipal water at 14±1°C. Test concentrations of Reward® were a geometric series of five concentrations with a targeted mid-range concentration of 0.37 mg/L, which is the predicted water concentration during Reward® applications to a water body according to Syngenta (Syngenta, 2015).

2.1.2. Animals

Eyed rainbow trout embryos were obtained from Troutlodge (Washington, USA) and transported to SFU in a specialized transport cooler chilled to 4 to 8°C with ice. Salmonid eggs were rinsed with diluted Ovadine (Syndel Canada; 5 mL Ovadine: 1 L dechlorinated municipal tap water) to ensure disinfection prior to being added to glass tanks for acclimation. Some eyed embryos to be used for studies later in development were transferred to Heath Stacks for rearing until 7 d post-hatch. Stock alvein fish were then moved to flow-through tanks and fed a commercial salmonid feed (Complete Fish Feed for Salmonids, EWOS Pacific, Surrey BC, Canada) twice daily until subsequent juvenile exposure experiments and toxicity tests.

2.1.3. Exposure Apparatus

Ten litre glass tanks were used for acclimatization and fish exposures. A overflow drainage hole of 2.5 cm diameter was drilled through each glass tank at the 6 L mark to allow for a flow through system. A plumbing connector was secured in the drilled hole allowing a drainage tube to be attached to the tank. Tank water was continually renewed with dechlorinated municipal tap water at 14 ±1°C delivered by a multiple head Masterflex L/S peristaltic pump (Cole-Parmer, USA) at a rate of 4.2 ml/min into each tank. A water holding tank was set up adjacent to the pump to facilitate a 4.2 ml/min flow to all tanks to allow one full water change per day. To maintain the recommended water temperature of 14 ±1°C four tanks were placed at random in 60 cm by 43 cm water baths filled with chilled water. Water baths were maintained at approximately 13±1°C by continual water flow from SFU Alcan de-chlorinated municipal water system set to maintain constant temperature. Figure 2.1 shows the exposure apparatus set-up.

2.1.4. In Vivo Rainbow Trout Exposures to Reward®

Three main experiments examining the effects of Reward® on rainbow trout were conducted and are described in separate Sections below.

Acute lethality 96 h rainbow trout exposure to Reward®

Two acute lethality toxicity tests were conducted on juvenile rainbow trout according to protocols outlined in *Biological Test Method: Acute Lethality Test Using*

Rainbow Trout (Environment Canada, 2007) to determine the concentration lethal to 50 % of exposed fish over a 96 h period (i.e., 96 h LC_{50}). These exposures are summarized in Table 2.1. The first acute toxicity test was performed on juvenile fish 56 d post-hatch to determine the sensitivity of these fish to Reward®, and to aid in determining concentration ranges for the subsequent juvenile pulse exposures. Nominal test concentrations of Reward® were 0, 0.37, 0.80, 1.8, 4.0, 8.7 mg/L. The second 96 h LC_{50} toxicity test was conducted in-order-to delineate mortality at higher concentrations and was conducted using fish 85 d post-hatch. Nominal test concentrations of Reward® were 0, 6.5, 10, 15, 22.5 mg/L for the second 96 h LC_{50} toxicity test. Exposure concentrations were prepared in clean 20 L food grade plastic buckets and diluted with dechlorinated municipal water at 14±1°C. Both 96 h LC_{50} tests were static exposures and water/Reward® test concentration renewals for each tank were conducted daily. Each test concentration was prepared in a dedicated 20 L food grade plastic bucket by pipetting concentrated Reward® into the appropriate volume of dechlorinated municipal water at 14±1°C.

Each concentration was tested in duplicate with seven fish per tank. Fish were not fed 16 h prior to the start of testing and were not fed during testing. Tank cleaning, water renewals and water quality monitoring (pH, temperature, dissolve oxygen, ammonia and conductivity) were conducted daily. A 1 L sample was collected from one replicate tank for the 6.5 mg/L treatment and one from a dechlorinated municipal water control tank and were submitted for water hardness and diquat ion analyses to Maxxam Analytics (Ste-Foy, Quebec, Canada). All samples were collected in plastic collection bottles, wrapped in tin foil and shipped on ice in a cooler to Maxxam Analytics.

Pre feeding early-life stage pulse dose exposure to Reward®

In vivo embryo pulse exposures to Reward® were conducted from the eyed embryo through to the pre-feeding swim-up fry developmental stage according to protocols outlined in the *Environment Canada Biological Test Method: Toxicity Tests Using Early Life Stages of Salmonid Fish; Rainbow Trout* (Environment Canada, 1998). To mimic Reward® application protocols for aquatic weeds, which limit multiple applications to every 14 d, fish were dosed for a 24 h exposure (i.e., a pulse exposure) followed by a 14 d period of non-exposure (i.e., reared in clean dechlorinated municipal water). The early life stage exposure was conducted for 26 d in total, and swim-up fry were harvested 10 d following the second pulse dose. The experiment duration, fish age and exposure are summarized

in Table 2.2. Pulse exposures were conducted in static conditions for the 24 h exposure period, however a flow-through system was used to maintain a water renewal rate of ≤0.5 g/L during non-exposure periods. Nominal test concentrations were 0.015, 0.074, 0.37, 1.85, 9.25 mg/L (diquat ion) plus a water control. All test concentrations were conducted in quadruplicate glass tanks, and each replicate tank contained 25 eyed embryos. Rainbow trout eyed embryos were allowed to acclimatise in tanks for 5 d prior to chemical exposure. Exposure concentrations were prepared in clean 20 L food grade plastic buckets and diluted with dechlorinated municipal water at 14±1°C.

Diquat ion concentrations in the test water were measured at the onset of exposure during the early-life rainbow trout exposures. A 1 L water sample was collected from each test concentration and the control from one randomly selected replicate tank. To test chemical stability, volatilization and adherence of diquat ion to the glass test vessels during a 24 h period, an additional glass tank containing the Reward® formulation and an aeration line in the absence of fish was prepared (i.e., volatilization test). Two water samples were collected from this tank: one at the onset of exposure period (0 h) and the second was collected 24 h later. A residual concentration sample was also collected from an exposure tank at one day post exposure to evaluate the effectiveness of tank flushing and evaluate potential residual concentrations during freshwater rearing. The residual sample was collected from a replicate tank in the high dose treatment group (9.25 mg/L). All samples were collected in clean 1 L containers supplied by Maxxam Analytics and were wrapped in tin-foil to reduce exposure to light and were stored in a cooler at 4°C for shipment and until analysis. All diquat samples were shipped on ice in a cooler to Maxxam Analytics (Ste-Foy, Quebec, Canada).

During the initial stages of the early life pulse exposure, the tests were conducted in the dark and one-week after the embryos hatched into alevins the photoperiod was 16 h of light and 8 h of darkness controlled by an automatic timer. Light intensity at the water surface was approximately 200 lux, which is within the recommended range of 100 to 500 lux (Environment Canada, 1998). Water quality was monitored daily using a HACH portable HQ40d multimeter to measure conductivity and temperature. The pH was monitored using a HACH Pocket Pro pH pen. Ammonia was monitored using a Multitest Ammonia Marine and Freshwater Kit (Seachem®, Madison, Georgia). Water parameters were monitored and compared to recommended limits for exposure as outlined in the guidance document for biological test methods using rainbow trout (Environment Canada,

1998). Behavioral observations were made daily prior to and during water quality monitoring, and tank cleaning which was typically a 1 h duration.

Juvenile feeding life stage pulse dose exposure to Reward®

In vivo juvenile pulse exposures to Reward® were conducted according to protocols outlined in the *OECD Guideline for the Testing of Chemicals, Fish Juvenile Growth Test Technical Guidance 215* (OECD, 2000). Pulse exposures conducted on juvenile rainbow trout were completed in the same 10 L glass tank flow-through apparatus as the early life stage exposures and as described in Section 2.1.3. At the start of the exposure, fish were approximately 1 g and 50 mm fork length at 66 d post hatch. Fish were fed a commercial salmonid feed (Complete Fish Feed for Salmonids, EWOS Pacific, Surrey BC, Canada) once daily at a rate of 4% of body weight (i.e., 0.04 g of feed per g of individual fish). A loading rate of 1.5 g/L was maintained in accordance with the loading rates (1.2 to 2.0 g/L) recommended by OECD guidance (OECD, 2000). Tanks were cleaned twice daily via siphons to remove waste. Nominal test concentrations were 0.12, 0.37, 1.1, 3.3, 10 mg/L diquat ion in Reward® plus a water control. All concentrations were tested in quadruplicate and each tank contained 7 fish. Juvenile trout were allowed to acclimatise for 5 d prior to chemical exposure.

During the juvenile rainbow trout exposures 1 L water samples were collected from the highest (10 mg/L) and lowest (0.12 mg/L) treatment groups from one replicate tank at the onset of exposure. Only high and low concentrations were analysed as full chemical analysis on all treatment groups was conducted during early-life exposures and showed consistency between nominal and exposure concentrations. All samples were collected in clean 1 L containers supplied by Maxxam Analytics and were wrapped in tin-foil to reduce exposure to light and were stored in a cooler at 4°C for shipment and until analysis. Water samples were sent to Maxxam Analytics (Ste-Foy, Quebec, Canada) for diquat ion analysis.

During exposures, the photoperiod was maintained at 16 h of light and 8 h of darkness controlled by an automatic timer. Light intensity at the water surface was approximately 200 lux, which is within the recommended range of 100 to 500 lux (Environment Canada, 1998). Water quality was monitored daily using a HACH portable HQ40d multimeter to measure conductivity and temperature. The pH was monitored using a HACH Pocket Pro pH pen. Ammonia was monitored using a Seachem Multitest Kit

Laboratories kit. Water parameters were monitored and compared to recommended limits for exposure as outlined in the guidance document for biological test methods using rainbow trout (Environment Canada, 1998). Behavioral observations were made daily prior to and during water quality monitoring, feeding, and tank cleaning which was typically a 1 hour duration.

2.1.5. Fish Euthanization and Tissue Collection

Fish from all exposures were euthanized using a lethal dose (0.4 g/L) of tricaine methanesulfonate (MS-222) adjusted to a pH of 7.0 to 7.4 with sodium bicarbonate (Sigma Aldrich, USA). A total of 443 fish were euthanized from the early life exposure at 26 d post hatch (total fish euthanized for each treatment group are: control 83; 0.015 mg/L, 80; 0.074 mg/L, 83; 0.37 mg/L, 78; 1.85 mg/L, 81; 9.25 mg/L, 38). A total of 168 fish (28 fish from each treatment group) were euthanized from the juvenile pulse exposure at 86 d post hatch. Fish were patted dry with paper towel and weight and fork-length were recorded for each individual fish. Deformity analysis was conducted for each fish upon termination using a graduated severity index (GSI) for assessing larval fish deformities, and this ranking system was based on methods established by Rudolph (2006). Briefly, the deformity analysis included evaluation of skeletal, craniofacial, finfold, and edema. The GSI index ranged from 0 to 3, with 0 indicating no deformity and 3 indicating a severe deformity (Rudolph, 2006). To ensure quality control of deformity assessment the GSI system was established prior to examination of samples and 10 % of ratings where re-examined by a second evaluator. In addition to these deformity assessments, the extent of yolk sac reabsorption was also noted for each fish. Whole livers from swim-up fry and juvenile rainbow trout were then collected using RNase/DNase free dissecting tools (tools were cleaned between each dissection with hydrogen peroxide and rinsed with RNase free water) under dissecting microscope. Livers were immediately placed in 1.5 ml RNase/DNase free tubes and flash frozen on dry ice; liver tissue was then placed at -80°C for storage.

2.1.6. Hepatic Tissue Protein Extractions

Protein extractions and proteomics were completed for the control and the 0.37 mg/L treatment groups for both the early-life stage and juvenile rainbow trout pulse exposure experiments. The 0.37 mg/L exposure concentration was selected because it

represents the predicted water concentration of diquat after a Reward® application to a body of water undergoing treatment for plant pests (Syngenta, 2015). Four individual biological replicates of extracted proteins were prepared for the control group and for the 0.37 mg/L exposure group. This was accomplished by randomly selecting one fish liver from each of the four replicate tanks and extracting hepatic protein. For the early life stage study three livers were combined (i.e., from the same exposure tank) prior to the tissue homogenization process to obtain sufficient protein quantity (100 µg) required for proteomic evaluation.

Proteins were extracted from fish livers using Ambion TRIzol™ Reagent (Sigma Aldrich, USA), according to manufacturers instructions (i.e., Invitrogen TRIzol™ method) Briefly, livers were homogenized in 1 mL of TRIzol™ using a micro-bead tissue homogenizer (MM 300 Tissuelyser Mixer Mill, Retsch, USA) and allowed to sit at room temperature for 5 min in order to lyse cells. Phase separation was established by the addition of 0.2 ml of chloroform (Anachemia, Lachine, Quebec, Canada) followed by a 15 min centrifuge at 12,000 x g at 4°C (Sorvall ST 16R Centrifuge, Thermo Fisher Scientific Inc., USA). The organic phase and interphase were collected and DNA precipitation was completed using 0.3 ml of ethanol (Commercial Alcohols, Brampton, Ontario, Canada). The supernatant was removed to a new tube and proteins were precipitated using 1.5 ml of isopropanol (Caledon Lab Chemicals, Georgetown, Ontario, Canada). The protein pellet was then washed twice with 2 ml of 0.3 M guanidine hydrochloride (Sigma Aldrich, Oakville, Ontario, Canada) in 95% ethanol. A final pellet wash was conducted with 2 ml of ethanol. The pellet was allowed to air dry for 5 min and was re-suspended in 1% sodium dodecyl sulfate (SDS) and an 8M urea solution (Sigma Aldrich, Oakville, Ontario, Canada). Proteins levels were quantified using a fluorescence Epoch 2 microplate spectrophotometer and a Take 3™ Micro-Volume Plate (BioTek, Winooski, Vermont, USA). The protein re-suspension solution was stored at -80°C.

2.1.7. Protein Quantification

The protein re-suspension solution was shipped on dry ice for 8-plex iTRAQ® quantitative proteomic assessment by University of Victoria (UVic) Genome BC Proteomics Centre, in Victoria, BC, Canada. At the UVic Genome BC Proteomics Centre protein samples were manipulated as follows according to UVic Genome BC Proteomics Centre iTRAQ® Quantitation Reports (personal communication, February 10, 2017).

Samples were quantified using bicinchoninic acid (BCA) assay and then were precipitated with acetone and re-solubilized in a triethylammonium bicarbonate (TEAB) and 0.2 % SDS solution. Proteins were then reduced with Tris (2-carboxyethyl) phospine hydrochloride (TCEP), alkylated with methyl methanethiosulfonate (MMTS) and digested with 80 µg trypsin (Promega, Sequencing Grade Modified Trypsin), a proteolytic enzyme used to cleave proteins into peptides. Prepared individual protein samples were then iTRAQ® labeled (AB Sciex, ON, Canada) and incubated for 2 hours at room temperature. The four individual control samples and four individual exposure samples were labelled with isobaric affinity labels (control labels 117, 118, 119 and 121; exposure labels 113, 114, 115 and 116) to allow for multiplexing of the 8 individual samples. Following labelling the eight samples were combined for separation by reversed phase high performance liquid chromatography (HPLC). Fractions from the HPLC column were collected every 1 min for 96 mins and fractions were analyzed by liquid chromatography mass spectrometer (LC-MS/MS). Data files were created by XCalibur 3.0.63 (Thermo Scientific) software and analyzed with Proteome Discoverer 1.4.0.228 software suite (Thermo Scientific). Proteins were searched against a teleost database in Uniprot (January, 2017). Proteome Discoverer result files were then analyzed using Scaffold 4.0 software for statistical validation of protein identifications.

The criteria for protein identification was used to validate MS/MS based peptide and protein identifications using Scaffold 4.0. Peptide identifications were accepted if they could be established at greater than 95 % probability by the Scaffold Local False Discovery Rate (FDR) algorithm. The FDR statistic measures the proportion of incorrect peptide identifications. Protein identification were accepted if they could be established at greater than 95 % probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al, 2013). 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 sharing significant peptide evidence were grouped into clusters.

For proteomic evaluation, a Benjamini-Hochberg permutation test for multiple test correction was applied with a significance level of $p<0.05$. Average fold change of the treatment group compared to the control group using all replicate sets was determined using Scaffold Software. Identified proteins were then sorted according to the following criteria; 1) a minimum fold change of 1.2 and, 2) a Benjamini-Hochberg significance value

of $p<0.05$. Gene codes for each protein were searched using GeneCards® Human Gene Database and UniProt Knowledgebase (October, 2017) search tools for use in pathway analysis.

2.1.8. Pathway Analysis

Pathway Analysis was conducted at Florida University, Center for Environmental and Human Toxicology College of Veterinary Medicine Department by Dr. Christopher Martyniuk. All proteins identified in iTRAQ® quantitative proteomic analysis for which gene symbols could be identified (~700) were imported into Pathway Studio using the official gene code for proteins. Interaction networks for differentially abundant proteins were built for early-life stage and juvenile protein datasets using Pathway Studio v11 (Elsevier). In Pathway Studio the option of "Highest magnitude fold change, and best p-value" was used as the default setting to accommodate any duplicated proteins in the dataset. Subnetwork enrichment analysis (SNEA) was conducted using protein fold change for "cell process", and "expression targets". SNEA analysis provides a list of differentially expressed proteins, genes, and pathways to be examined between exposure and control animals. The relationships are built upon co-expression patterns, binding, or involvement in common pathways focused on gene hubs.

2.1.9. Statistical Analysis

Probit Analysis was used to calculate a 96 h LC_{50} concentration according to protocols outlined in *Guidance Document on Statistical Methods for Environmental Toxicity Tests* (Environment Canada, 2005). To evaluate differences in growth, survival and deformity among treatment groups for early-life and juvenile pulse dose rainbow trout exposures a one-way Analysis of Variance (ANOVA) and Tukey's post-hoc test ($p<0.05$) was conducted using JMP Statistical Software (SAS Institute Inc. 2012). For proteomic evaluation, a Benjamini-Hochberg permutation test for multiple test correction was applied with a significance level of $p<0.05$. Average fold change of the treatment group compared to the control group using all replicate sets was determined using Scaffold Software. For the pathway analysis, a Mann-Whitney U test was used to determine whether or not sub-networks were significantly ($p <0.05$) different compared to a background distribution in the program.

2.2. Results

2.2.1. Water quality and pesticide exposures

Water temperature was set to maintain a constant temperature of 14±1°C as recommended in the *Environment Canada Biological Test Method: Toxicity Tests Using Early Life Stages of Salmonid Fish; Rainbow Trout* (Environment Canada, 1998). The water temperature during all exposures ranged from 13.3°C to 15.0°C with an average temperature of 14.1°C. The pH value remained consistent and ranged from 7.2 to 7.8 with an average value of 7.4. Conductivity ranged from 24.2 to 37.3 $\mu S/cm^2$ with an average value of 24.9 $\mu S/cm^2$ during freshwater and exposure periods. Conductivity was highest for the highest concentration treatment group during exposures (average 35.9 µS/cm) likely due the commercial formulation components or ionic nature of the chemical. Ammonia levels were below the 5 µg/L recommended maximum value. Water hardness of municipal dechlorinated water used in exposure tests was determined to be approximately 10.5 mg/L.

Water chemistry samples were analysed by Maxxam Analytics in Ste-Foy, Quebec, Canada. Nominal values were similar to the measured values and are listed in Table 2.3. The largest difference between nominal and measured values was for the early-life stage exposure (treatment level 0.37 mg/L) which represents the typical environmental exposure to fish (nominal 0.37 mg/L, measured 0.27 mg/L). A volatilization test was conducted to examine if diquat ion concentrations in water (no fish present) would change over a 24 h period in the presence of an aeration line. The volatilization test sample was collected at the onset of the test (0 h), and the diquat ion concentration was 8.0 mg/L. The sample collected at 24 h of aeration had a concentration of 8.1 mg/L. The volatilization test showed no change in tank concentration after 24 h of aeration with no fish present, indicating that the chemical did not volatilize or adhere to the tank glass over a 24 h period. A residual test was also conducted during the Reward® fish exposure experiment to determine if any residual chemical remained in tanks following a water change after the 24 h exposure period. This sample was collected from one replicate tank following a 24 h pulse exposure to Reward® and once the tank had been renewed with freshwater. The residual test showed the diquat ion concentrations were less than detection limits (<0.07 mg/L) in tanks following renewal to the clean water flow-though system, indicating fish

were not exposed to Reward® during this 14 d non-exposure period. Analytical results are summarized in Table 2.3.

2.2.2. Acute Lethality Toxicity Test

Two acute toxicity 96 h LC_{50} tests were conducted on juvenile rainbow trout. The first 96 h LC_{50} ranged in test concentrations from 0.37 to 8.7 mg/L and resulted in 43% mortality for the highest treatment group. The second 96 h LC_{50} test was conducted to examine mortality at higher concentrations and to determine the lethal concentration at 50% mortality (i.e., LC_{50}). Diquat concentrations for the second 96 h LC_{50} ranged from 6.5 to 22.5 mg/L and the concentration-response curve is shown in Figure 2.2. Probit Analysis was conducted on the second 96 h LC_{50} test to calculate an LC_{50} concentration of 9.8 mg/L. Percent mortality for both toxicity tests, and the probit curve is shown in Supplemental Information (A1-A2).

During the second 96 h toxicity test observations of fish showed lethargy, loss of equilibrium and operculum movement appeared to increase in Reward® exposed fish compared to control fish at all concentrations (6.5 to 22.5 mg/L). At high doses (22.5 mg/L) 100 % mortality was achieved at 50 h post exposure. For 15 mg/L treatment group 73 % mortality was achieved at 96 h and remaining fish were not swimming, near the bottom of the tank, and showed loss of equilibrium effects (i.e., upside-down, sideways or with nose pointed down).

2.2.3. Pre Feeding Early Life Stage Pulse Dose Exposure to Reward®

Survival data for pre-feeding early-life stage pulse exposure fish are shown in Figure 2.3a. For the early-life stage exposure, mean percent survival was highest for the control group (94 % ± 3 SE) and ranged from 89 % ± 5 to 92 ± 3 % mean survival for the 0.015 to 1.85 mg/L treatment groups. However, the highest-level treatment group of 9.25 mg/L showed significantly reduced mean survival rates (43 % ± 4 SE). The length and weight of early-life stage rainbow trout exposed to two pulse doses of Reward® are shown in Figure 2.4a and 4b. For the early-life stage exposure there was a significant decrease in mean length between the control and highest treatment group (9.25 mg/L; $p = 0.0011$; Figure 2.4a). Similarly, a significant decrease in mean weight between the control and 9.25 mg/L treatment group ($p = 0.043$) was observed.

Few fish deformities were observed at the conclusion of the exposures (data not shown). Of the 443 fish terminated in the early-life exposure, only 7 fish had deformities which were evident upon hatch. For the highest treatment group (9.25 mg/L), one fish had mild lordosis of the spine (GSI score 1). For the 1.85 mg/L treatment group, two fish had spinal deformities; one fish with severe kyphosis (GSI score 3) and the other fish with severe scoliosis (GIS score 3) along with mild edema of the eye (GSI score 1). For the 0.37 mg/L treatment group, two fish had mild edema of the eye (GSI score 1) and one of those fish also had a mild craniofacial deformity in the form of a slight jaw malformation (GSI score 1). For the 0.015 mg/L treatment group two fish had deformities. One fish had a mild craniofacial deformity in the form of a jaw overbite (GSI score 1) and severe yolk sac edema (GSI score 3), the other fish had mild craniofacial deformity in the form of an underbite (GSI score 1) and had a reduced tail. There were no significant differences in deformities among treatment groups (i.e., p values <0.05) and deformities likely reflect natural background deformities as opposed to those caused by chemical treatment.

During the early-life stage pulse exposure after the second 24 h pulse exposure in the 9.25 mg/L treatment group, 9 of the 38 fish remaining among all replicate tanks (24 %) were observed to be lethargic, not swimming, and had little or no avoidance at capture. One of the 9 fish was observed upside down and most fish at the treatment level were observed to have reduced feeding. The survival rate for this treatment group was 43 % ± 4 SE at termination which included a return to freshwater for 10 d, after which, the fish showed no sign of recovery.

Proteomics

Proteomics data were evaluated using Scaffold 4.7.5 Software. Identified hepatic proteins that met the established criteria for significance value ($p<0.05$) and fold change (minimum of 1.2) compared to the control treatment were evaluated further and are discussed in subsequent sections, however pathway analysis was conducted using all identified proteins. Full protein lists are provided in Supplemental Data. Proteomic results for the early life stage experiment represent an average fold change value from three biological replicates. A total of 1,207 hepatic proteins were identified for the early-life stage exposure, and 315 of those proteins were significantly different between the control and 0.37 mg/L diquat ion (Reward®) treatment group (i.e., met the established criteria for p-value and fold change).

The majority of identified proteins with expression changes significantly different from the control group are known to be involved in cellular biosynthetic processes (18 %, e.g., glycogen synthase), RNA binding activities (14 %, e.g., RNA binding motif protein), catalytic functions (10 %, e.g., Triosephosphate isomerase), and ATP binding functions (10%, e.g., Myosin IB). Other identified proteins are involved in developmental processes (7 % e.g., Valosin containing protein) and stress response (4 %, e.g., Heat shock protein 5). The distribution of Gene Ontology (GO) annotations of biological process and functions for identified hepatic proteins for early life stage rainbow trout are shown in Figure 2.5. Of the 315 proteins that were statistically different from in the 0.37 mg/L diquat ion treatment group compared to the control, 140 proteins were downregulated and 175 proteins were upregulated relative to the control. A selection of upregulated and downregulated hepatic proteins with the highest fold change are shown in Figure 2.6. Downregulated proteins with highest fold change from control, included Pantothenate kinase 4 (-13.6), Cell division cycle 42 (-10.3), Serine/threonine-protein phosphatase (-5.4), Calcium-transporting ATPase (-4.7). Upregulated proteins with highest fold change include Alpha-tropomyosin (3.5), beta Tropomyosin 2 (2.7), Parvalbumin 4 (2.7), Desmin b (2.6), Creatine kinase muscle isoform 2 (2.6).

Pathway Analysis

Subnetwork enrichment analysis was conducted using Pathway Studio (Elsevier, 2017) on the full suite of proteomics data (i.e., 1,207 hepatic proteins) and was used to evaluate cellular process and expression targets for the early life stage dataset. A total of 55 cellular processes and 70 expression targets were identified to have hepatic protein expression changes in the Reward® (0.37 mg/L diquat ion) exposed swim-up fry compared to the water controls. Table 2.4 shows the cellular process, major themes, and fold change and p-values. For example, the SNEA pathway data indicated that the sterol regulatory element binding protein (SREBP) pathway was downregulated for the early life stage rainbow trout (fold change -1.3). The SREBP is a transcription factor that regulates the glycolysis and lipogenesis pathways in the liver. The pathway SREBP is involved in is shown in Figure 2.7. In addition, SNEA revealed that the cellular process of splice site selection (fold change 1.7; Figure 2.8), protein splicing (fold change 1.7; Figure 2.9) and mRNA metabolism (fold change 1.4; Figure 2.9) increased significantly in the livers of Reward® exposed swim-up fry. Finally, the CASP3 pathway was upregulated in the Reward® exposed swim-up fry (fold change 1.4; Figure 2.10). The CASP3 protein is a

member of the cysteine-aspartic acid protease (caspase) family, and the activation of caspase plays a central role in cell apoptosis or cellular death (Zeng, 2014).

Cellular processes related to immune system were decreased for exposed fish such as platelet response (fold change -1.4), platelet function (fold change -1.2), and blood flow (fold change -1.1). Calcium mobilization was increased for exposed fish (fold change 1.2). Protein kinase C beta type (PRKCB) was significantly increased (fold change 1.7) and is activated by calcium and involved in a diverse number of cellular signaling pathways. Ubiquitin-protein ligase E3A (UBE3A) was significantly increased (fold change 1.6) and is an enzyme implicated in protein degradation. Ubiquitin protein ligase E3A attaches a small marker protein called ubiquitin to proteins that should be degraded. Cellular structures called proteasomes recognize and digest proteins tagged with ubiquitin. Atypical chemokine receptor 3 (ACRK3) was significantly decreased (fold change (-1.6) and is a cytokine signalling protein. Some chemokines are considered pro-inflammatory and can be induced during an immune response to recruit cells of the immune system to a site of infection, while others are considered homeostatic and are involved in controlling the migration of cells during normal processes of tissue maintenance or development (Qi, 2015).

2.2.4. Juvenile Feeding Life Stage Pulse Dose Exposure to Reward®

Survival data for juvenile-life stage pulse exposure are shown in Figure 2.3b. For the juvenile exposures no mortality occurred for any of the treatment groups. For the juvenile pulse Reward® exposure experiment, there was a significant difference in mean length between the 10 mg/L and 3.3 mg/L treatment groups ($p = 0.038$), but not at lower concentrations (Figure 2.11a). For weight, there was a significant increase between the control and the 0.12 mg/L, 1.1 mg/L and 3.3 mg/L treatment groups (p values: 0.048. 0.022, and 0.042, respectively; Figure 2.11b). No fish deformities were observed in the juvenile pulse exposures. Fish exposed to environmentally relevant concentrations (0.37 mg/L) and lower concentrations showed no behavioral abnormalities. At higher doses (3.3 to 22.5 mg/L) fish showed reduced feeding behavior, lethargy, increased operculum movement, and loss of equilibrium.

Proteomics

Proteomics data were evaluated using Scaffold 4.7.5 Software. Identified hepatic proteins that met the established criteria for significance value ($p<0.05$) and fold change (minimum of 1.2) were evaluated further and discussed in subsequent sections. Full protein lists are provided in Supplemental Data. A total of 987 hepatic proteins were identified for the juvenile exposure and 84 proteins met established criteria for p value (<0.05) and fold change (1.2). The majority (42 %) of identified proteins are involved in cellular biosynthetic processes (e.g., Aldehyde dehydrogenase 4 family, member A1) or are identified ribosome constituents (20 %; e.g., Ribosomal protein L9). Other identified proteins are involved with RNA, actin or calcium ion binding, transport, microtubule process or oxidation reduction. The distribution of Gene Ontology (GO) annotations of biological process and functions for identified hepatic proteins for juvenile Rainbow Trout are shown in Figure 2.12.

Of the 84 proteins examined, only six identified proteins were downregulated, in the liver of Reward® exposed fish compared to control fish, while remaining proteins were upregulated. A selection of upregulated and downregulated hepatic proteins with the highest fold change are shown in Figure 2.13. Downregulated proteins and fold change include; Dynactin 1a (fold change -1.56), Calreticuline (fold change -1.22), Prohibitin (fold change -1.22), High density lipoprotein binding protein a (fold change -1.22), Nascent polypeptide-associated complex subunit alpha (fold change -1.21) and Cathepsin D (fold change -1.20). The majority of these proteins act as binding proteins for different cellular processes. Dynactin 1a is involved in microtubule process as a dynein complex binding protein, Calreticuline is involved in calcium ion binding, Prohibitin is a lipid binding protein and high density lipoprotein binding protein a is an RNA binding protein.

The majority of identified hepatic proteins were upregulated for exposed fish compared to controls. The four highest upregulated proteins included Transducin (beta)-like 2 (fold change 1.68), Rab GDP dissociation inhibitor (fold change 1.52), ARP3 actin related protein 3 homolog (fold change 1.52), and S-(hydroxymethyl)glutathione dehydrogenase (fold change 1.46). Transducin (beta)-like 2 protein is involved in cellular response to glucose starvation and hypoxia and molecular functions including protein kinase binding and phosphoprotein binding. Rab GDP dissociation inhibitor protein is involved in many processes including protein transport, small GTAase mediated signal

transduction, molecular function- GTPase activator activity, oxidoreductase activity, and RAB GDP-dissociations inhibitor activity. The main function of Rab GDP dissociation inhibitor protein is regulation of the GDP/GTP exchange reaction of most RAB proteins by inhibiting the dissociation of GDP and inhibiting the subsequent binding of GTP. The ARP3 actin related protein 3 encodes the ARP2/3 complex located in the surface of the cell which is thought to be essential to the motility and shape of the cell. S-(hydroxymethyl) glutathione dehydrogenase is an enzyme belonging to the oxidoreductases family.

Pathway Analysis

Subnetwork enrichment analysis was conducted using Pathway Studio (Elsevier, 2017) on the full suite of proteomic data and was used to evaluate cellular process and expression targets for the juvenile life stage datasets. A total of 38 cellular processes and 28 expression targets were identified to have expression changes for exposed juvenile life stage fish compared to controls. Table 2.5 show the cellular process, major themes and fold change and p-value. The majority of cellular processes (92 %) and expression targets (96 %) with significant expression changes increased from the control group. The only expression target downregulated for the juvenile dataset is the cystic fibrosis transmembrane conductance regulator (CFTR) which has evidence of expression related to changes in water salinity. The sterol regulatory element binding protein (SREBP) is a transcription factor that regulates the glycolysis and lipogenesis pathways in the liver. The SNEA pathway data indicates that the SREBP pathway is upregulated for the juvenile stage rainbow trout (fold change 1.3). The pathway is shown in Figure 2.7.

The CASP3 protein is a member of the cysteine-aspartic acid protease (caspase) family. The activation of caspase plays a central role in cell apoptosis or cellular death. The CASP3 pathway is upregulated for the exposed juvenile rainbow trout (fold change 1.0). The serine/threonine-protein kinases (Atk kinases) mammalian target of rapamycin (mTOR) pathway is involved in regulation of the cellular cycle including cellular growth, and survival. There are three main Atk kinases isoforms active in liver tissue including Atk1, Atk2, and Atk3. In juvenile exposed fish the Atk1 and mTOR showed an expression change (fold change 1.08 and 1.09, respectively) from control groups. The ATK pathway is shown in Figure 2.14. Glycogen degradation was increased in exposed juvenile fish (fold change 1.2). The glycogen degradation pathway is shown in Figure

2.15. Regulation of translational fidelity (fold change 1.3) and ribosome biogenesis and assembly (fold change 1.1) are significantly increased for exposed fish. These pathways are shown in Figure 2.16.

2.3. Discussion

The objective of this research was to examine the acute toxicity and sub lethal effects of a commercial formulation of an aquatic herbicide, Reward®, on multiple early life developmental stages of rainbow trout. The acute toxicity experiments indicated a 96 h LC_{50} of 9.8 mg/L of diquat ion using this commercial formulation on rainbow trout aged 85 d post-hatch. This toxicity value is similar to values presented previously for exposures conducted using the pure active ingredient (diquat ion) on rainbow trout of varying and unknown ages. Although environmental concentrations of diquat are not measured in Canada, Syngenta claims that the water concentration of diquat ion is 0.37 mg/L after aquatic applications of Reward® to treat pest aquatic plants, and that concentrations are expected to dissipate to 0.01 mg/L within 24 h. Although no effects on growth and development of alevins were observed after two 24 h pulse applications (0.37 mg/L diquat ion) of Reward®, separated by a two week non-exposure period, sub-cellular expression changes on the hepatic proteome were evident in both pre-feeding swim-up fry and in feeding fry. Hepatic proteome effects were more dramatic in the pre-feeding swim-up fry with 315 proteins significantly different between the control and fish exposed to Reward®, while in the later life stage feeding fry, only 84 proteins were significantly different after Reward® exposure. This study is the first to report the sub-cellular and whole organism level effects of this commercial formulation of Reward® and demonstrates that the abundance of specific proteins can change at environmentally relevant concentrations based on aquatic application rates.

2.3.1. Reward® Effects on Growth, Development and Survival

Direct comparison to the limited acute toxicity data available from previously reported 96 h LC_{50} studies in rainbow trout is challenging due to limited study details (e.g., fish age, water hardness) reported in the scientific literature. Additionally, previous acute toxicity studies were largely conducted using the active ingredient only (i.e., diquat ion), not a commercial formulation (i.e., Reward®) as reported in the present study. However,

the 96 h LC_{50} value determined in this study of 9.8 mg/L of diquat ion is within the range of previously reported values for rainbow trout ranging from 9.5 mg/L to 16 mg/L (Pimentel et al, 1971; US EPA, 1995; Campbell et al, 2000; Emmett, 2002). One study reported in the *Washington State Department of Ecology Final Risk Assessment for Diquat Bromide* (Emmett, 2002) conducted on rainbow trout fingerlings (no age/size reported) indicated a 96 h LC_{50} of 9.5 mg/L (hardness not specified), 15 mg/L (hardness <50 mg/L), and 14.9 mg/L (hardness >50 to 150 mg/L). A second study reported in this same risk assessment, also conducted on rainbow trout fingerlings (~ 50 mm length) reported that the 96 h LC_{50} was 16 mg/L (hardness >50 to 150 mg/L). Emmett (2002) reported that under hard water conditions the toxicity of diquat is decreased by up to ten-fold, however specific studies were not included. In the present study, the water hardness for the acute exposure was 10.5 mg/L $CaCO_3$ indicative of soft-water conditions, and although the fish in the present study were slightly larger in size (~60 mm) compared to those reported for Emmett (2002), the trend of softer water increasing toxicity of the diquat ion appears to be supported. Future studies examining water hardness as a toxicity modifying factor for diquat dibromide and commercial formulations containing this active ingredient are warranted to elucidate the extent of water hardness in attenuating toxicity at both the whole organism and sub-organism level.

It is widely accepted that early life stages of vertebrates can exhibit increased sensitivity after exposure to xenobiotics compared to adult life stages, and this appears to be further supported in the present study. Survival rates after two 24 h pulse exposures to Reward® showed that the early-life stage pre-feeding swim-up fry were more sensitive to Reward® (43 % ± 4 survival; 9.25 mg/L) compared to juvenile feeding fish (aged 66 to 86 d post hatch; 100% survival rates at 10 mg/L). The early life stage pre-feeding fry also showed decreased body morphometrics after 9.25 mg/L Reward® exposures, whereas no decreases in these measures were observed at a similar concentration in the feeding fry (10 mg/L diquat ion). However, at concentrations ranging from 0.12 to 3.3 mg/L neither pre-feeding or feeding fry exhibited adverse effects with respect to survival, body length or weight. Collectively, applying two pulse applications of the commercial formulation Reward® according to the manufacturer's instructions, reveal no immediate effects on survival and body morphometrics in early life stages of rainbow trout. Currently environmental concentrations of diquat are unknown and are not monitored under the National Pesticides Monitoring and Surveillance Network program (Environment and

Climate Change Canada) that reports annual concentrations of other commonly used pesticides. If application protocols are not followed during aquatic use, actual concentrations could be higher than the target application concentration or could be increased by cumulative effects such as runoff from terrestrial applications. In addition, these studies only monitored animals for ~24 hs after these Reward® pulse exposures, so whether latent/delayed adverse effects on survival and body morphometrics would ensue is unknown and longer observation periods in future studies is recommended.

2.3.2. Proteome Response and Underlying Subnetworks after Pulse Dose Reward® Exposure

In this study, 1,207 hepatic proteins were identified for the early-life stage pre-feeding swim-up fry and 987 proteins were identified for feeding juvenile rainbow trout. Unique hepatic proteome responses to 0.37 mg/L diquat dibromide after Reward® exposures were evident in these two life stages. A total of 33 identified proteins were found to be in common between the pre-feeding swim-up fry and the feeding fry. After two 24 h pulse exposures of Reward® (0.37 mg/L diquat ion) separated by 14 d in clean water, 315 proteins were significantly different compared to the controls in the pre-feeding swim-up fry, while only 84 hepatic proteins were significantly different between the control and Reward® exposed liver of feeding fry. Interestingly, most of the identified proteins (93 %) in the liver of the older feeding juvenile fish exhibited increased expression relative to the controls, while liver protein expression in the pre-feeding swim-up fry relative to the control fish were both up- and downregulated (55 % upregulated and 45% downregulated). A variety of proteins involved in several common biological processes were significantly affected in the liver of both life stages of rainbow trout after Reward® exposures including, calcium ion binding, actin binding, transport, RNA binding ribosome constituent, oxidation reduction and cellular biosynthetic processes. While some biological processes with significant changes in protein targets were unique to pre-feeding swim-up fry including developmental process, ATP binding, catalytic activity, stress response, protein catabolic process, GTP binding, and structural molecular activity. Overall, these results suggest significant changes in cellular protein levels ensue after two single applications of the aquatic herbicide Reward® separated by 14 d, and that this response is dramatically different during rainbow trout early life stage (embryonic/alevin/swim-up fry) exposures compared to feeding fry.

Although no studies examining proteome changes after exposure to commercial formulations of Reward® are reported in the literature, a similar study on juvenile rainbow trout showed expression changes of ~140 genes 24 h after a 500 µg/kg intraperitoneal injection of pure diquat (Hook et al, 2006). Hook et al. (2006) found that the majority of gene expression changes were mainly increased in abundance in these juvenile rainbow trout (5-7 months/1,870-2,460 degree days old) rather than down-regulated. The genes that were upregulated were involved in transport, nucleic acid binding, oxidoreductase activity, protein binding, calcium ion binding, and ATP binding processes which are similar processes identified in the present study in both life stages tested. Hook et al. (2006) noted higher fold change values for gene expression changes (ranging from 1.5 to 25) which may be representative of the higher dose. Lower protein expression fold changes in this study (ranging from 1.0 to 1.7) for feeding fry may also be reflective of a recovery period for fish as euthanasia took place 5 d after the second pulse exposure. However, how absolute or fold change levels for transcripts and proteins correlate is poorly understood for most targets. Studies examining the common gene and protein changes after Reward® exposure in the present study compared to Hook et al. (2006) would be useful next steps for examining mode of action of diquat as well as correlating transcript and protein expression levels. Hook et al. (2006) also exposed fish to chromium (VI), known to cause oxidative stress, and found that 96 % of genes were upregulated. The juvenile feeding fry in the present Reward® exposure study were ~ 2-3 months of age and showed a similar trend of a higher number of upregulated proteins (78 out of 84 proteins altered after Reward® exposure, or 93%), suggesting that in juvenile fish upregulation of transcription and translation may be associated with oxidative stress. Although in the pre-feeding early life stage exposure only 55% were upregulated (173 out of 315 proteins altered by Reward®), it is likely that proteins underlying early life stage development were affected as well as those associated with oxidative stress but this requires further testing.

The bidirectional protein expression changes in the present study after Reward® exposures in the earlier life stage pre-feeding swim-up fry compared to the unidirectional upregulation observed in the feeding fry may also indicate different toxic modes of action of diquat in these different developmental stages. Additional evidence of different proteome responses to Reward® exposure in these two life stages in the present study are the unique individual protein response patterns and many biological processes identified by the SNEA analyses. These results are likely due in some part to differences

in liver development between the early pre-feeding swim-up fry and feeding fry during and upon termination of the Reward® exposures. Hinton et al. (2004) suggested that metabolism of the yolk for the sac-fry is linked to the formation of the bile in the embryonic fish. In the second week of larval life Iwamatsu et al. (2003) reported that for *Oryzias latipes* a metamorphosis occurs when the yolk sac is completely absorbed, which includes elongation of the gut and re-positioning of the liver in the abdominal cavity. During this metamorphosis and re-positioning of the liver, the hepatic portal vein is established allowing blood flow to the liver (Hinton et al, 2004). In the present study, at the end of the early-life stage pulse Reward® exposures, alevin yolk sacs were completely re-absorbed (i.e., yolk sac rating of 0) for the majority of fish (80 %), however fish may have had internal yolk sac remnants. It is possible, and quite likely, that since this study was terminated at the onset of the swim-up fry developmental stage that the livers were not fully functioning or formed and the final positioning of the hepatic portal may not have been fully complete. This final maturation and development of the gut and liver was complete in the later life stage feeding fry in the present study, thus, the more dynamic up- and down-regulation pattern of the proteome response in the pre-feeding swim-up fry is likely due, in some part, to the complex and poorly understood developmental process transitioning from a non-feeding larva to a feeding fish.

Generally, both the pre-feeding swim-up fry and feeding juvenile fish after two 24 h pulse exposures separated by 14 d in clean water showed signs of increased processes related to RNA and protein development and/or processing in the liver. In particular in juvenile fish enriched subnetworks of proteins related to RNA processes, including ribosomal biogenesis and assembly, translational fidelity and rRNA processing, were significantly increased. This suggests RNA and proteins were being developed at a higher rate for exposed fish. Although the implications on whole organism adverse outcomes is not fully understood, ribosome biogenesis and assembly is a complex process that can lead to improper protein synthesis if affected (Marjan et al, 2017). RNA and protein related processes were also significantly increased for early-life stage pre-feeding swim-up fry, however unique processes were identified between the two life stages in the present study. For pre-feeding fry these RNA processes included: mRNA metabolism, RNA binding, ribonucleoprotein complex assembly, polyadenylation, protein splicing and splice site selection. Increases to RNA processes and protein synthesis may be indicative of a response to cellular injury or disruptions in regular cellular processes, and other studies

have noted expression changes to ribosomal genes in response to oxidative stress (Afonso et al, 2003; Thorpe et al, 2004; Hook et al, 2006). This further supports the notion that differences between pre-feeding and feeding fry were evident, yet some of the commonalities in protein subnetworks appear to be associated with oxidative stress.

The process of glycogen degradation was also significantly increased in liver of juvenile feeding fry after pulse dose exposure to 0.37 mg/L of diquat ion in the Reward® formulation compared to the control fish. Glycogenolysis, or the breakdown of glycogen into glucose, takes place in the liver and muscle cells (Hilton et al, 2008). The breakdown of glycogen in hepatocytes delivers glucose into the blood stream for use by other cells; this process typically occurs in response to energy depletion in the body caused by cellular stress or physiological conditions such as starvation (Engelking, 2015). Glycogenolysis is regulated by glucagon and insulin levels in the body (Hilton, 2008). Glycerol biosynthesis by pyruvate was also significantly increased for juvenile fish in the present study. The synthesis of glycerol by pyruvate is part of the glyceroneogenesis metabolic pathway in the liver, which regulates lipid levels in the body (Hilton et al, 2008). In the present study lipogenesis was upregulated in the feeding juvenile fish along with lipid export. Interestingly, effects related to these cellular processes that increased in the juveniles were also altered in the pre-feeding swim-up fry, such that the ultimate effects may have caused similar increased free glucose and decreased lipid degradation. Specifically, for the pre-feeding early life stage swim-up fry, the cellular process of insulin release was downregulated along with lipid degradation. In hepatocytes, an increase in insulin-specific signaling is correlated with stimulation of glycogen synthesis and/or inhibition of gluconeogenesis, while a decrease would result in glucose not being utilized for these processes and more free glucose available in the cell. However, opposing effects in the two different life stages in the present study were observed for the SREBP pathway, which was increased in juvenile feeding fry and downregulated for pre-feeding fry. The SREBP is a transcription factor that regulates the glycolysis and lipogenesis pathways in the liver (Horton et al, 2003). A recent study conducted by Ruiz et al, 2013 indicated that SREBP and isoforms also play an important role in regulation of carbohydrate metabolism by promoting glycogen synthesis, enhancing glycolysis and inhibiting gluconeogenic gene expression in fed mice. In the present study juvenile fish activation of the SREBP pathway suggests that the downstream effect of lipogenesis was activated, while it was inactivated in pre-feeding swim-up fry. Nohturfft and Zhang (2009) suggested that SREBPs are

activated to produce lipids required for repair or development of membranes, which can be damaged during oxidative stress. This inability to produce lipids for repairing damaged membranes due to oxidative stress supports the notion of reduced metabolic/detoxification capabilities in immature animals compared to more mature animals. Nonetheless, a different mode of toxic action of diquat in developing fish compared to more mature fish cannot be ruled out.

The Atk pathway regulates cellular processes including protein synthesis, energy metabolism and apoptosis. In juveniles this expression target was upregulated in the liver compared to controls along with TORC1. The activation of the Atk pathway, in turn, activates the mTOR pathway (Porta et al, 2014). When mTORC1 is activated it initiates the synthesis of many proteins and is related to increased mRNA translation (Porta et al, 2014). Luu et al. (2011) suggested that Atk is involved in the activation of the SREBP pathway. Additionally, CASP3 expression was increased for both pre-feeding and juvenile feeding fry in the present study. The activation of caspases plays a central role in apoptosis or the cellular death cascade. The activation of the Atk/mTOR pathway, along with caspase activation suggests the occurrence of apoptosis in the liver of exposed fish. Increased hepatocyte death may be a result of toxic mode of action such as oxidative stress, or an indication of direct hepatoxic effect of the diquat on liver tissue. Further studies on hepatocyte injury following diquat exposure of varying doses and duration should be conducted to determine the extent of hepatotoxicity of diquat, and if it is severe enough to induce adverse effects in the whole animal.

For pre-feeding swim up fry exposed to two pulse exposures of Reward®, hepatic expression changes to cell processes related to the immune system were significantly downregulated including platelet response, formation and function, neutrophile chemotaxis, erythrocyte differentiation, and blood flow. The effectiveness of the immune response is also dependant on available energy reserves, and the Reward® exposed pre-feeding fry showed increases in the energy homeostatic response biological process pathway. In contrast, in the feeding fry some immune processes were upregulated including somatic hypermutation, a cellular mechanism involved in the adaptation of the body system to foreign elements indicating an immune response to a foreign element. In general, the SNEA for pre-feeding fry indicated a more varied immune system response than the juvenile feeding fry, whereby effects were more indicative of increases to most cellular processes in the feeding fry including immune processes. The downregulation of

multiple immunity processes for pre-feeding fry may indicate that this early life stage fish were less able to adequately respond to a stressor or toxicant, and may be related to an immature immune system in this pre-feeding early life stage (Marjan et al, 2017).

2.4. Conclusion and Future Work

In conclusion, this study demonstrated lethal concentrations and sub-lethal proteomic effects on two early life developmental stages of rainbow trout exposed to Reward®. Although no effects on growth and development of alevins were observed after two 24 h pulse applications (0.37 mg/L diquat ion) of Reward®, separated by a two week non-exposure period, sub-cellular impacts on the hepatic proteome were evident in both pre-feeding swim-up fry and in feeding fry. Hepatic proteome effects were more dramatic in the pre-feeding swim-up fry with 315 proteins significantly different between the control and Reward® exposed fish, while in the later life stage feeding fry only 84 proteins were significantly different after Reward® exposure. Although many unique cellular processes and biological pathways were effected after Reward® exposures in these two life stages, some commonalities were evident. Proteins involved in the oxidative stress and immune response pathways were effected in both life stages. Atk/mTOR signalling was increased in feeding fry which is implicated in cellular death cascades along with lipid regulation and nutrient signalling in the liver. Caspase activation occurred in both life stages, suggesting the occurrence of apoptosis in the liver of exposed fish indicating possibly hepatoxicity after Reward® exposure. Lastly, strong evidence of effects on metabolism in both life stages after Reward® exposure were observed based on significant changes in SREBP, a transcription factor that regulates the glycolysis and lipogenesis pathways in the liver. Based on the findings in the present study, future studies may include evaluation of hepatic injury through histopathological analyses of liver tissue, energy homeostasis/reserve indicators (e.g. blood glucose, glycogen levels), antioxidant enzyme levels as indicators of oxidative stress along with gene, protein and whole organism level effects on growth, development and survival. In addition, future experimental designs should incorporate a longer observation period after environmentally relevant exposures to examine chronic and/or latent effects and the potential whole organism adverse outcomes of the proteome wide changes observed in the present study.

Together, the results of the present study provide novel toxicity and proteomics data for diquat dibromide in a commercial herbicide formulation after environmentally

relevant exposure scenarios. In particular, this study generates valuable insights into the cellular pathways and biological processes to focus subsequent studies on in order to elucidate a toxic mode(s) of action of this chemical. In addition, this study significantly contributes to the global efforts aimed at establishing adverse outcome pathways by describing the sequential chain of causally linked events at different levels of biological organisation that lead to adverse whole organism or ecotoxicological effects. This study is the first to report the sub-cellular and whole organism level effects of this commercial formulation of Reward® and demonstrates that numerous changes at the protein level occur at environmentally relevant concentrations based on aquatic application rates.

2.5. Tables

Table 2.1 Summary of Rainbow Trout Acute Toxicity Tests (96 h LC_{50}) to Reward® Landscape and Aquatic Herbicide

Exposure	Duration (d)	Fish Age during exposure (days Post Hatch)	Exposure Description
96 h LC_{50}	4	57-61	Static
96 h LC_{50}	4	87-90	Static

Table 2.2 Summary of Rainbow Trout Pulse Exposures to Reward® Landscape and Aquatic Herbicide

Exposure	Duration (d)	Fish Age during exposure (days Post Hatch)	Exposure Description
Early Life (embryo to swim-up fry)	26	0-26	2 x 24 h pulse exposure periods 14 d apart, with fish reared in clean water in between pulses.
Juvenile	18	66-86	2 x 24 h pulse exposure periods 14 d apart, with fish reared in clean water in between pulses.

Table 2.3 **Nominal and measured concentrations of diquat ion during acute and sub-chronic rainbow trout exposures to Reward®.**

Experiment	Sample Type	Nominal (mg/L)	Measured (mg/L)
Early life-stage	Test vessel water	9.0	8.5
	Test vessel water	1.9	2.2
	Test vessel water	0.37	0.27
	Test vessel water	0.07	0.071
	Test vessel water	0.01	0.098
	Test vessel water	0	<0.07
Water Diquat Dissipation	Water - volatilization test onset	9.0	8.0
	Water - volatilization test - post 24 h	9.0	8.1
	Water - residual test	0	<0.07
Juvenile	Test vessel water	10	12
Juvenile	Test vessel water	0.12	0.12
96 h LC_{50}	Fish tissue	water 6.5	N/A[1]
96 h LC_{50}	Fish tissue	water 6.5	N/A[1]
96 h LC_{50}	Test vessel water	6.5	5.3
96 h LC_{50}	Test vessel water hardness	N/A	10.5

Note: [1] fish tissue samples collected during 96 h LC_{50} exposure were lost in a sample preparation error by Maxxam Analytics.

Measured values were assessed on 1 L samples by Maxxam Analytics (Le Foy, Quebec, Canada). Test vessel water measured the concentration of diquat ion at the onset of the 24 h pulse dose exposure and was collected from the test vessel containing fish during testing. The volatilization test was conducted on a tank containing no fish to evaluate potential dissipation of diquat ion over a 24 h period. The water residual test measured diquat ion in a test vessel containing fish following a 24 h pulse exposure and after the tank had been flushed with freshwater.

Table 2.4 **Early life stage rainbow trout sub-network enrichment analysis (SNEA) for cell process in livers collected from water controls versus Reward® (0.37 mg/L diquat ion) exposed fish in two 24 h pulse exposures separated by rearing in clean water (n=3 biological replicates). Major cell process theme, gene set seed, number of neighbors, fold change and p-value are presented (Pathway Studio v11, Elsevier).**

Major Theme	Gene Set Seed	# of Total Neighbours	# of Measured Neighbors	Fold Change	p-value
RNA/mRNA Process	RNA binding	322	60	1.27	0.0206
	mRNA processing	327	49	1.32	0.0335
	stress granule assembly	87	17	1.35	0.0327
	mRNA metabolism	113	27	1.38	0.0064
	polyadenylation	229	34	1.42	0.0338
	ribonucleoprotein complex assembly	31	10	1.45	0.0247
	mRNA 3'-end processing	31	9	1.52	0.0329
Cellular Processes	splice site selection	112	23	1.72	0.0000
	membrane invagination	62	8	-1.80	0.0037
	post Golgi transport	30	8	-1.45	0.0475
	plasma membrane repair	30	5	-1.26	0.0407
	cell phagocytosis	83	11	-1.25	0.0392
	caveolae-mediated endocytosis	41	8	-1.44	0.0454
	calcium mobilization	894	48	1.21	0.0182
	cell spreading	834	63	1.09	0.0226
	calcium metabolism	99	6	1.40	0.0266
	lipid degradation	638	47	-1.06	0.0392
	lipid absorption	101	9	1.18	0.0273
	epidermal cell differentiation	315	22	1.12	0.0494
Immune system/blood systems	platelet response	101	9	-1.44	0.0068
	platelet shape change	47	7	-1.44	0.0119

Major Theme	Gene Set Seed	# of Total Neighbours	# of Measured Neighbors	Fold Change	p-value
	neutrophil chemotaxis	304	17	-1.14	0.0189
	thrombocyte aggregation	526	38	-1.14	0.0083
	blood flow	588	30	-1.14	0.0431
	platelet function	355	32	-1.25	0.0145
	erythrocyte differentiation	563	42	1.12	0.0468
Growth/Muscle Process	muscle contraction	343	23	-1.04	0.0017
	growth hormone release	264	18	-1.24	0.0131
	prenatal growth	39	5	1.51	0.0139
	muscle fiber development	354	30	1.16	0.0216
	muscle function	528	41	1.09	0.0249
	postnatal development	593	33	-1.16	0.0322
	sarcomere organization	112	12	1.16	0.0330
	convergent extension	126	9	-1.44	0.0437
Nerve Process	actin myosin interaction	42	7	1.72	0.0180
	intron retention	55	14	1.69	0.0241
	axon extension	188	8	-1.11	0.0335
	dendritic extension	34	6	1.46	0.0434
	neuron homeostasis	83	6	1.51	0.0498
	vesicle docking	115	10	-1.54	0.0248
	nerve development	256	21	-1.14	0.0284
	Schwann cell formation	32	6	-1.25	0.0414
Cardio Process	heart relaxation	46	7	1.72	0.0007
	cardiomyocyte differentiation	199	14	1.16	0.0226
Protein	protein splicing	31	15	1.69	0.0039

Major Theme	Gene Set Seed	# of Total Neighbours	# of Measured Neighbors	Fold Change	p-value
	protein aggregation	272	39	1.28	0.0234
Biological Process	energy homeostasis	763	49	1.04	0.0422
	insulin release	1386	112	-1.14	0.0463
Other	acrosome reaction	229	17	-1.25	0.0372
	viral particle maturation	38	13	-1.03	0.0177
	reference memory	89	7	1.37	0.0320
	ectopic expression	404	22	1.32	0.0320
	kidney filtration	132	12	-1.22	0.0230
	stomach function	70	5	1.51	0.0294

Table 2.5 **Juvenile life stage rainbow trout sub-network enrichment analysis (SNEA) for cell process in livers collected from water controls versus Reward® (0.37 mg/L diquat ion) exposed fish in two 24 h pulse exposures separated by rearing in clean water (n=4 biological replicates). Major cell process theme, gene set seed, number of neighbors, fold change and p-value are presented (Pathway Studio v11, Elseivier).**

Major Theme	Gene Set Seed	Total # of Neighbours	# of Measured Neighbors	Fold Change	p-value
RNA/mRNA Process	rRNA processing	223	22	1.16	0.0278
	RNA duplex unwinding	6	5	1.24	0.0405
	DNA fragmentation	695	46	1.09	0.0458
	mitochondrial DNA depletion	50	7	1.28	0.0287
Cellular Process	cell respiration	140	20	1.15	0.0230
	lipogenesis	835	54	1.09	0.0222
	cell morphogenesis	182	16	1.04	0.0134
	cell junction assembly	47	9	-1.11	0.0152
	distribution of mitochondria	52	7	-1.06	0.0494
	clathrin-mediated endocytosis	343	38	1.08	0.0424
	ciliary motility	182	5	1.24	0.0423
	protein synthesis	1797	171	1.08	0.0151
Immune system/blood systems	somatic hypermutation	110	11	1.15	0.0138
	drug susceptibility	470	37	1.07	0.0202
	macrophage apoptosis	303	20	1.11	0.0206
	cell invasion	2683	151	1.05	0.0498
Growth/Muscle Process	growth regulation	652	45	1.06	0.0450
	smooth muscle development	87	7	1.14	0.0272
Nerve Process	neuroprotection	910	48	1.06	0.0358
	neurite outgrowth	1740	100	1.05	0.0481
Biological Process	glycogen degradation	126	14	1.23	0.0049

Major Theme	Gene Set Seed	Total # of Neighbours	# of Measured Neighbors	Fold Change	p-value
	ribosome biogenesis and assembly	350	38	1.12	0.0144
	physiological stress	79	7	1.05	0.0176
	glycerol biosynthesis from pyruvate	24	5	1.25	0.0311
	hemolysis	229	21	1.13	0.0180
	microtubule cytoskeleton organization	552	44	1.06	0.0453
	cytolysis	468	28	1.08	0.0218
	mitochondrion organization and biogenesis	464	35	1.09	0.0369
	lipid export	169	14	1.05	0.0443
Other	regulation of translational fidelity	35	11	1.27	0.0030
	dendritic spine morphogenesis	91	7	1.09	0.0081
	sperm capacitation	89	8	1.18	0.0162
	UV protection	44	5	1.13	0.0170
	spermatogenesis	1224	69	1.06	0.0334
	hatching	165	12	1.15	0.0173
	megakaryopoiesis	225	13	1.15	0.0331
	plasma cell differentiation	136	5	-1.06	0.0387
	skin barrier	211	8	1.15	0.0400

2.6. Figures

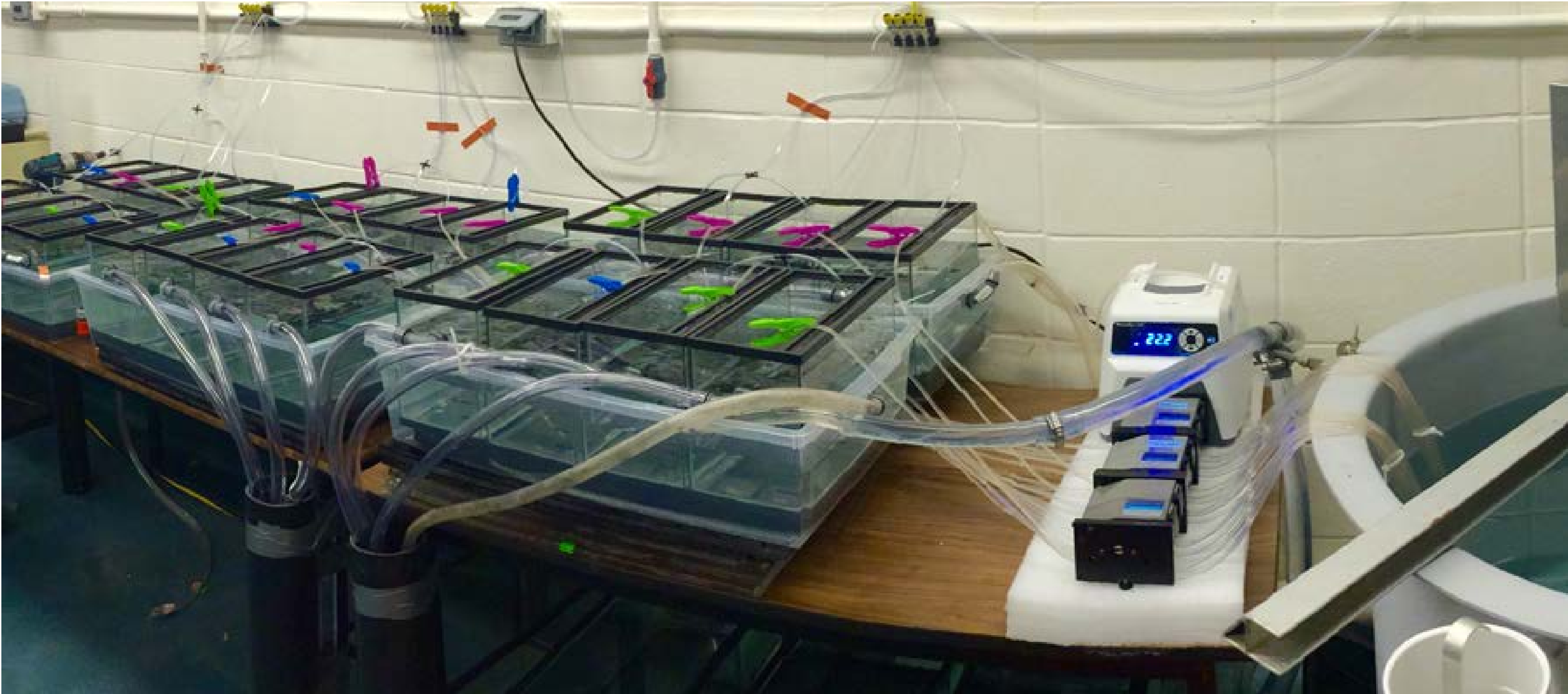


Figure 2.1 Rainbow Trout exposure apparatus set-up. Peristaltic pump shown on the right pumping freshwater from reservoir tank (far right) to the exposure tanks (left). Overflow drainage tubes are attached to exposure tanks by connector at the 6 L mark in order to enable the flow through system. A supplied airline was used to supply bubbled air to each tank.

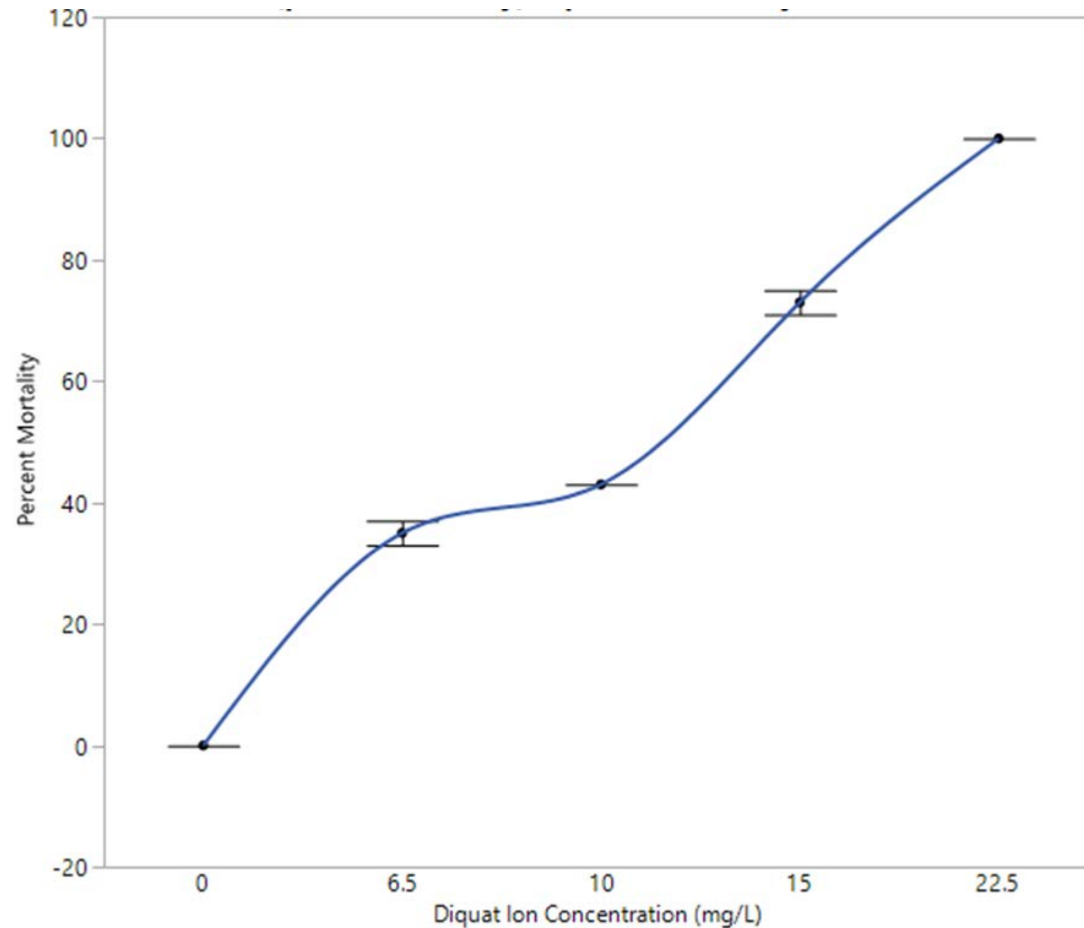
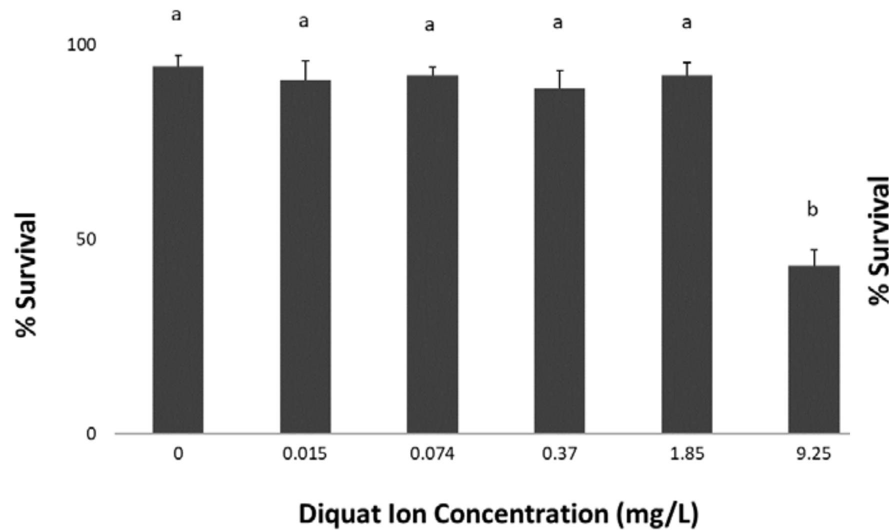


Figure 2.2 **Concentration response curve for the continuous 96 h LC_{50} toxicity test exposure to Reward® Landscape and Aquatic Herbicide conducted on rainbow trout 87 d post-hatch. Probit analysis was used to calculate a 96 h LC_{50} value of 9.8 mg/L, see supplimental Information A1-A3. Exposures were conducted in duplicate with 7 fish per tank. Error bars show standard error of the mean for each treatment group (n=2).**



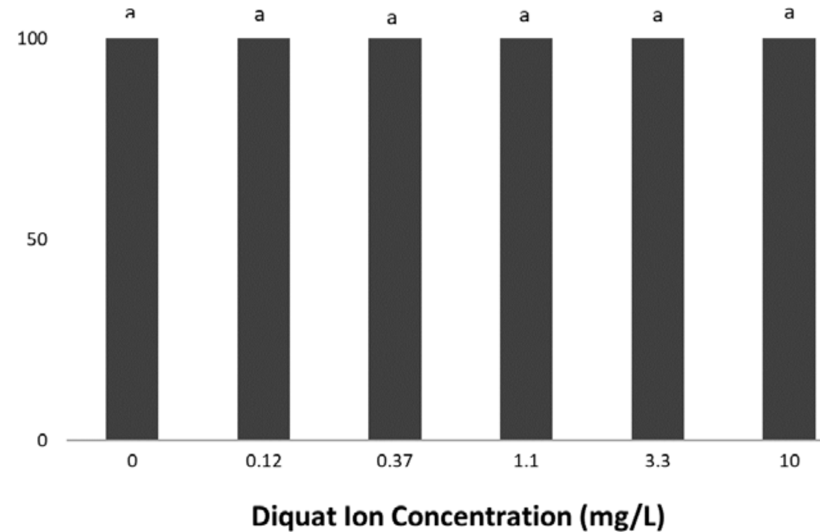


Figure 2.3 **The effects of rainbow trout following two 24 h pulse exposures to Reward® 14 d apart on survival for A) early-life stage (embryo through to swim-up fry) and B) juvenile fish (66-86 d post hatch). Values presented are means ± standard error (early life: n=4 tanks per treatment with 25 fish/tank, Juvenile: n=4 tanks per treatment with 7 fish/tank). Different letters indicate significant difference between treatments (one-way analysis of variance followed by a Turkey's host-hoc, $p<0.05$). No fish mortality occurred in the juvenile study (B) for any of the treatment groups. Exposure treatments were made up with Reward® Landscape and Aquatic Herbicide concentrate (240 g/L).**

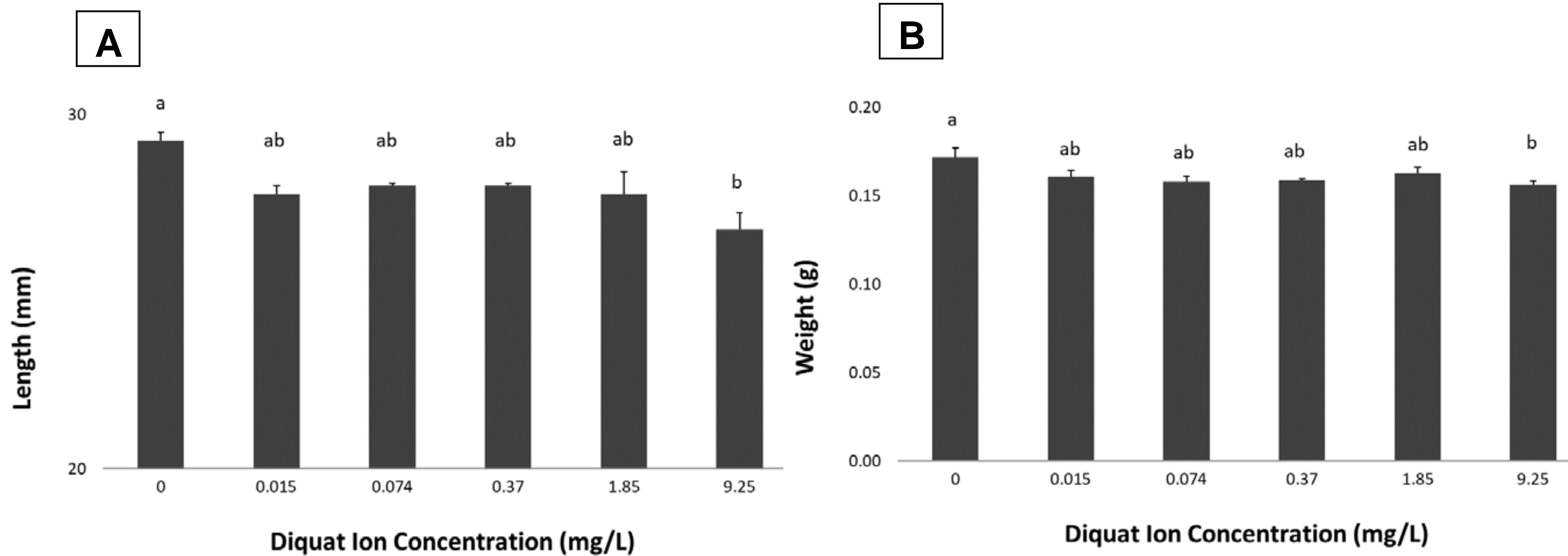


Figure 2.4 **The effects of early life stage rainbow trout (embryo through to swim-up fry) following two 24 h pulse exposures to Reward® 14 d apart on A) length and B) weight (average of each replicate; total wet weight). Values presented are means ± standard error (n=4 tanks per treatment with 25 fish/tank). Different letters indicate significant difference between treatments (one-way analysis of variance followed by a Turkey's host-hoc, $p<0.05$). Exposure treatments were made up with Reward® Landscape and Aquatic Herbicide concentrate (240 g/L).**

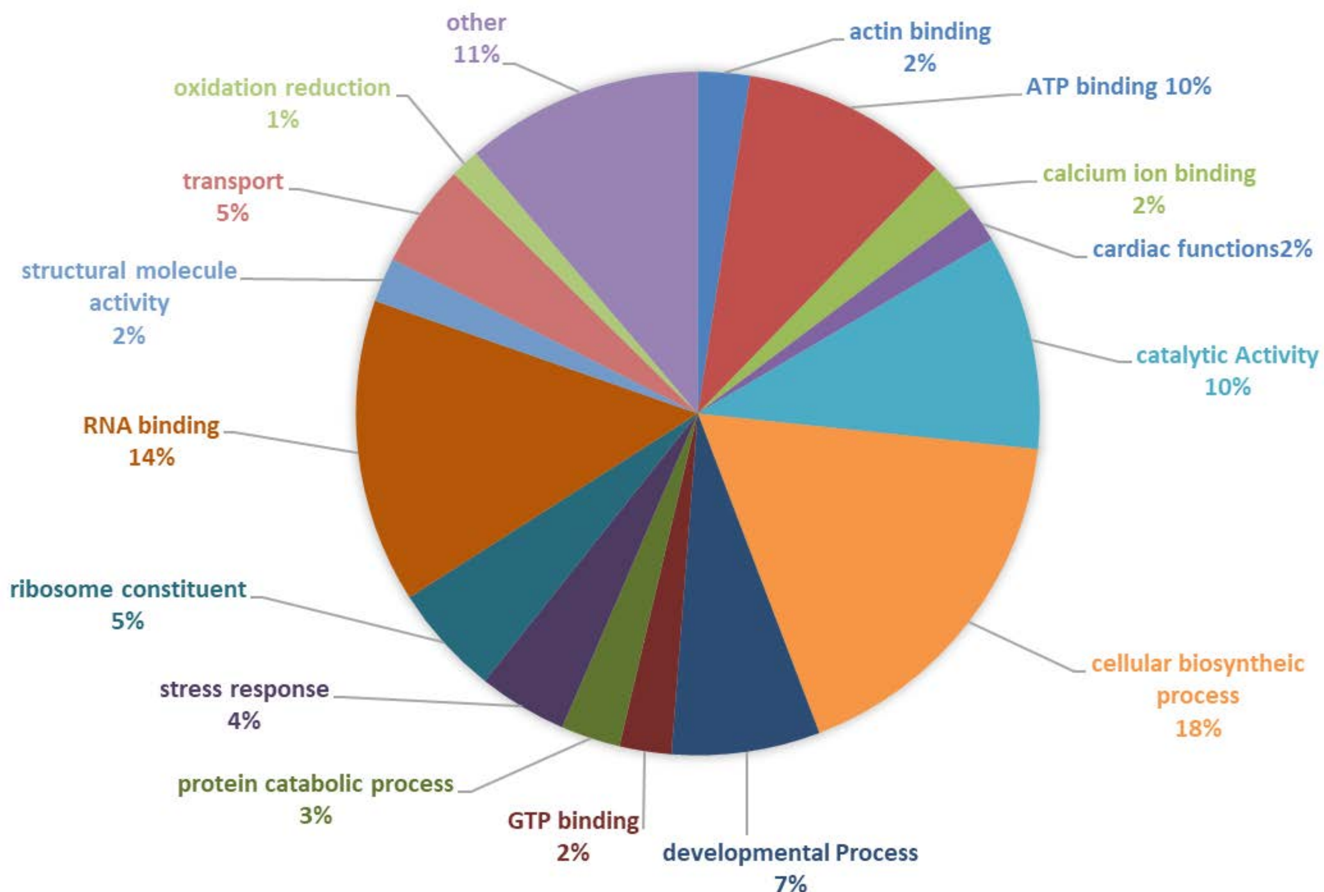


Figure 2.5 **Percent distribution of Gene Ontology (GO) annotations of biological process and functions for identified hepatic proteins with significant expression changes from treatment fish vs controls for early life stage rainbow trout (embryo to swim-up fry). Exposures to early life stage rainbow trout included two 24 h pulse exposures to Reward® Landscape and Aquatic Herbicide 14 d apart. If fewer than three proteins were identified for an annotation, then it is not displayed. Pie charts were constructed using Microsoft excel.**

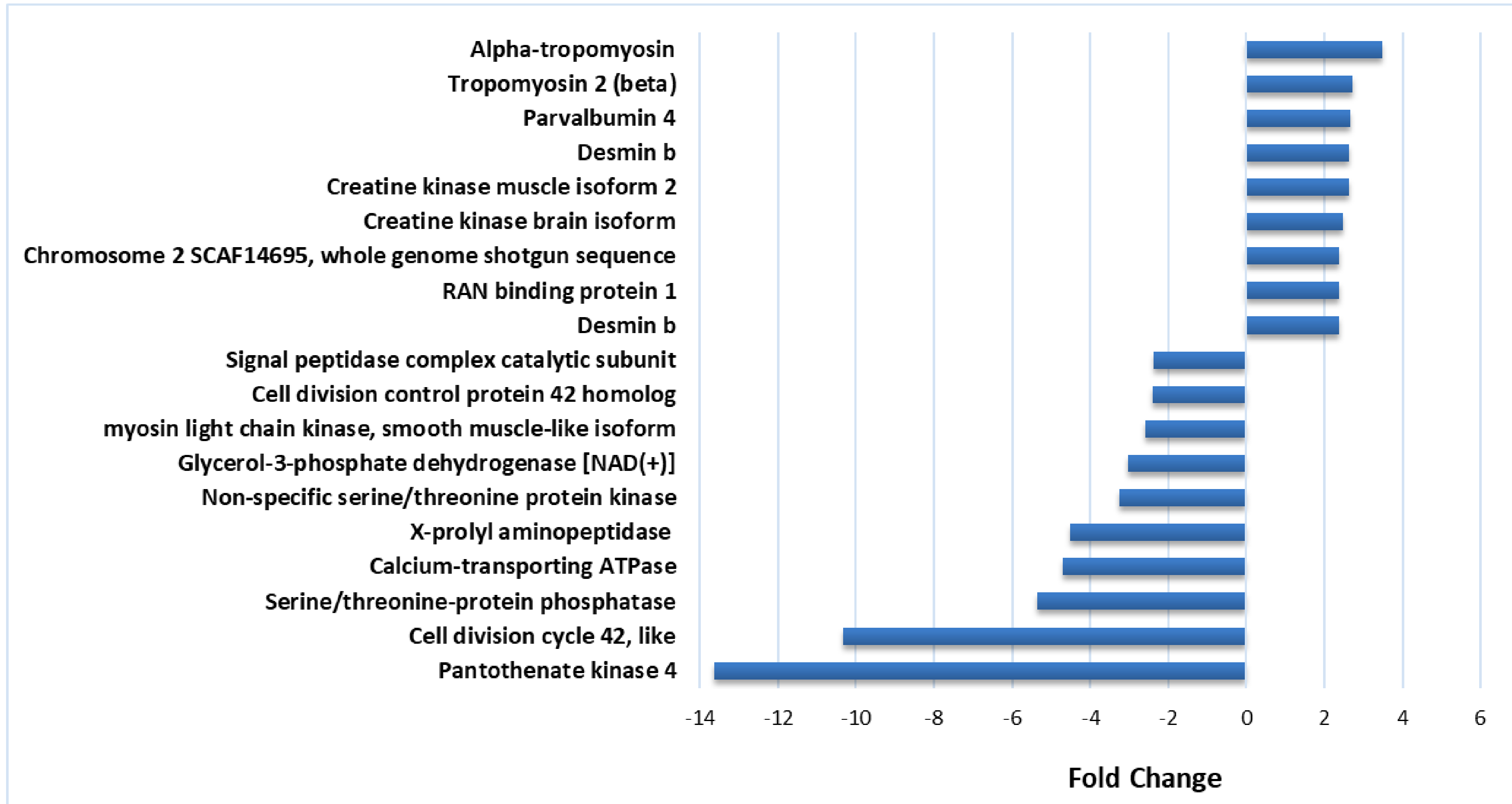


Figure 2.6 Variations in the expression of hepatic proteins after early-life stage rainbow trout exposure to diquat dibromide using Reward® formulation. The 10 significantly upregulated and downregulated hepatic proteins with highest fold change are shown relative to the water control. Data were analyzed using Scaffold 4 Software and a Benjamini-Hochberg correction was applied to determine significance ($p=<0.05$).

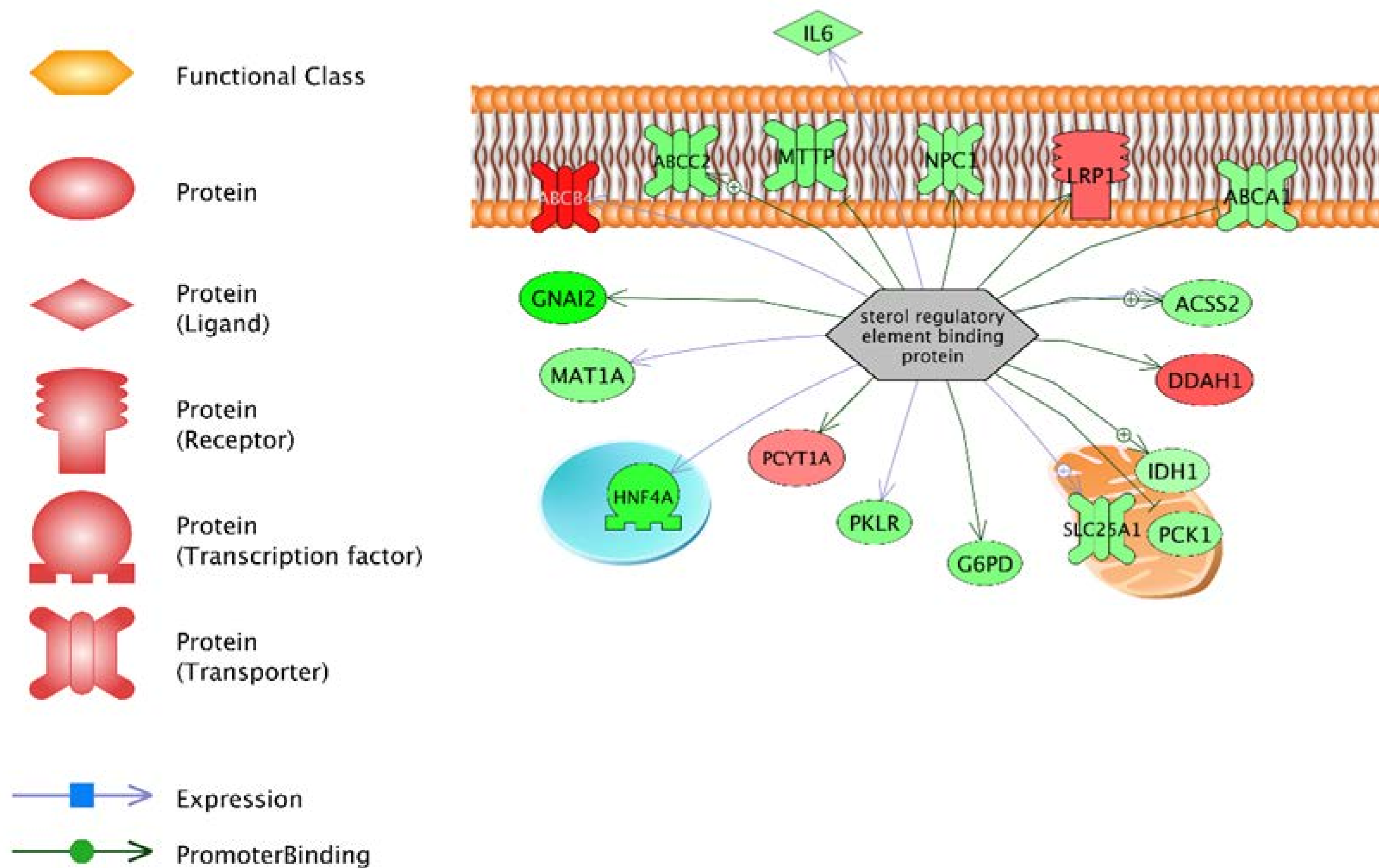


Figure 2.7 **Pathway studio analysis for protein network for differentially expressed proteins with the process of sterol regulatory element binding protein following diquat pulse exposures to early-life stage during the eyed embryo to swim-up fry developmental stage in rainbow trout. Red indicates an increase in relative protein levels while green indicates a decrease in relative protein levels. Abbreviations follow that given in Supplemental Data.**

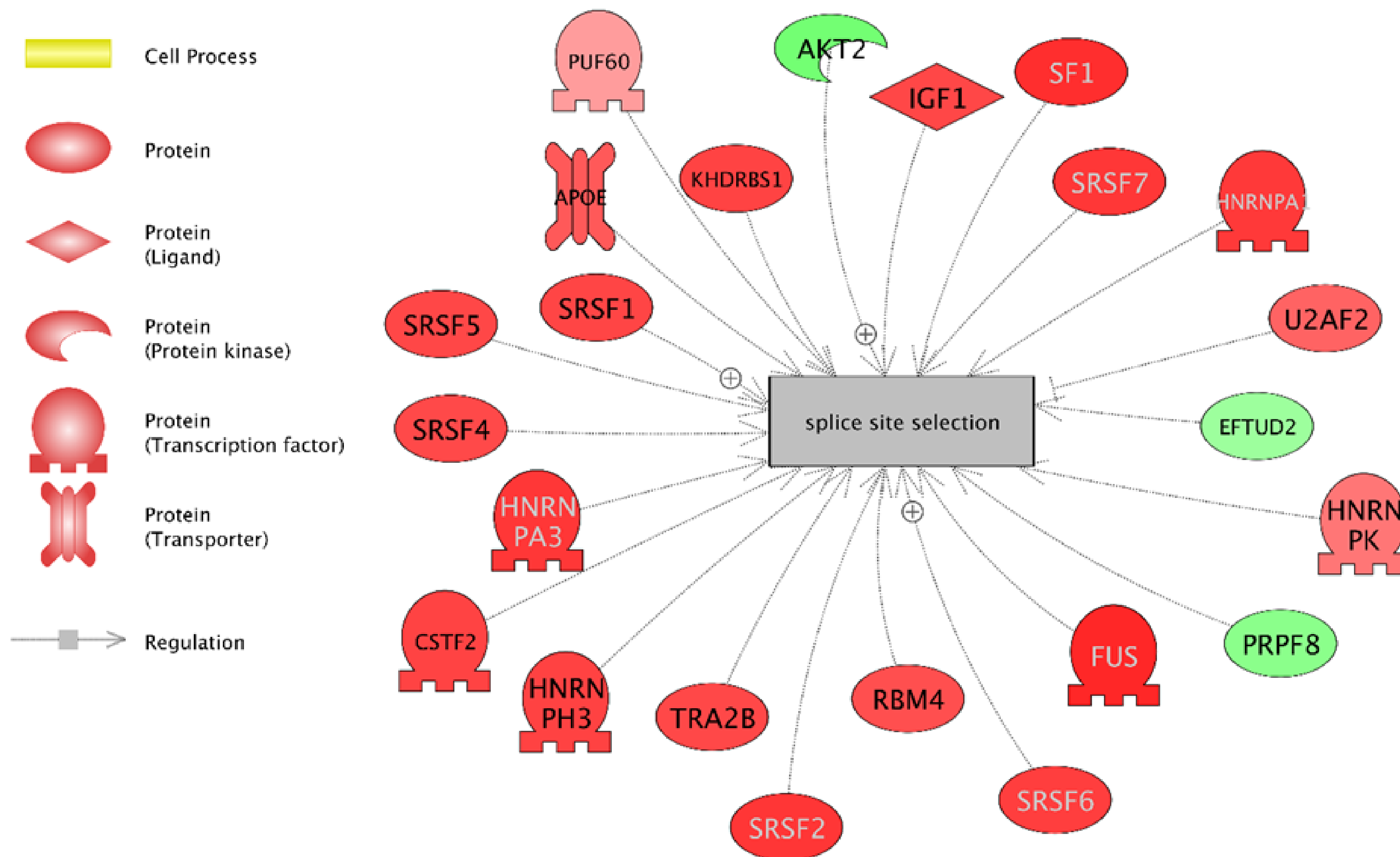


Figure 2.8 **Pathway studio sub-network enrichment analysis for differentially expressed proteins with the process of splice site selection following two 24 h pulse dose exposures of Reward® Landscape and Aquatic Herbicide to early-life stage rainbow trout (embryo to swim-up fry). Red indicates an increase in relative protein levels while green indicates a decrease in relative protein levels. Abbreviations follow that given in Supplemental Data.**

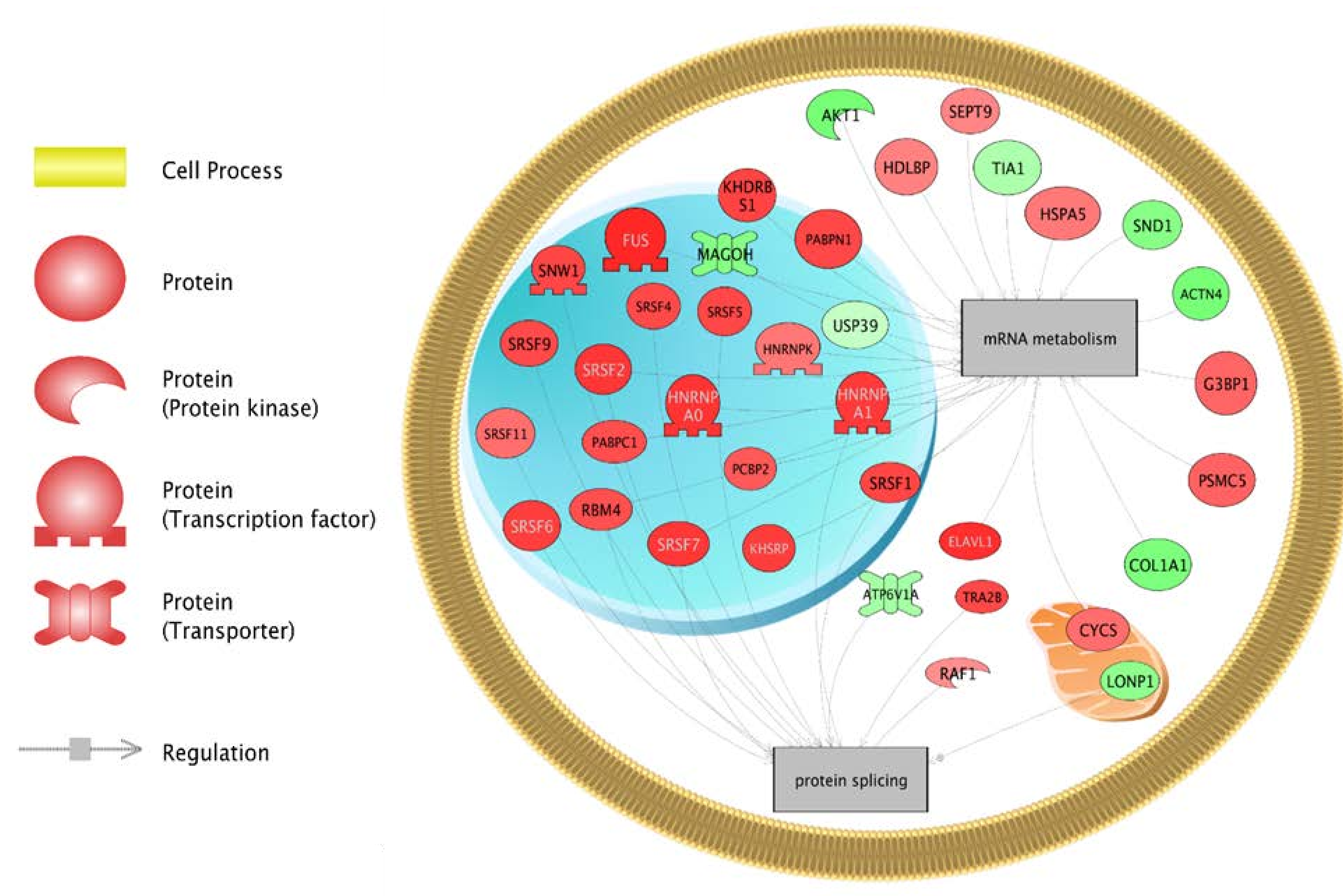


Figure 2.9 Pathway studio sub-network enrichment analysis for differentially expressed proteins with the process of mRNA metabolism and protein splicing following two 24 h pulse dose exposures of Reward® Landscape and Aquatic Herbicide to early-life stage rainbow trout (embryo to swim-up fry). Red indicates an increase in relative protein levels while green indicates a decrease in relative protein levels. Abbreviations follow that given in Supplemental Data.

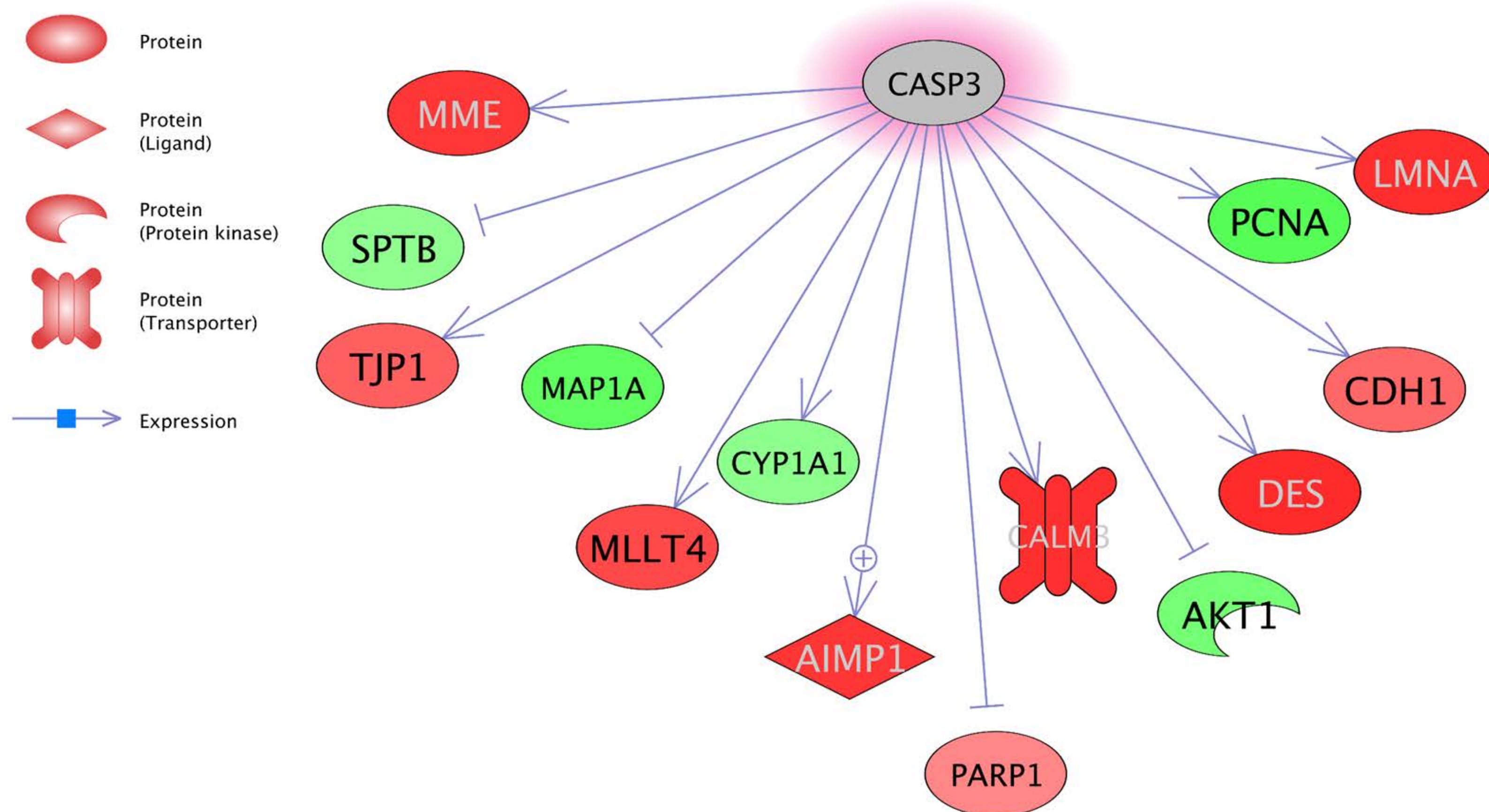


Figure 2.10. Pathway studio sub-network enrichment analysis for differentially expressed proteins with the master gene regulator CASP3 following two 24 h pulse dose exposures of Reward® Landscape and Aquatic Herbicide to early-life and juvenile stage rainbow trout (embryo to swim-up fry; 66-86 d post hatch, respectively). Red indicates an increase in relative protein levels while green indicates a decrease in relative protein levels. Abbreviations follow that given in Supplemental Data.

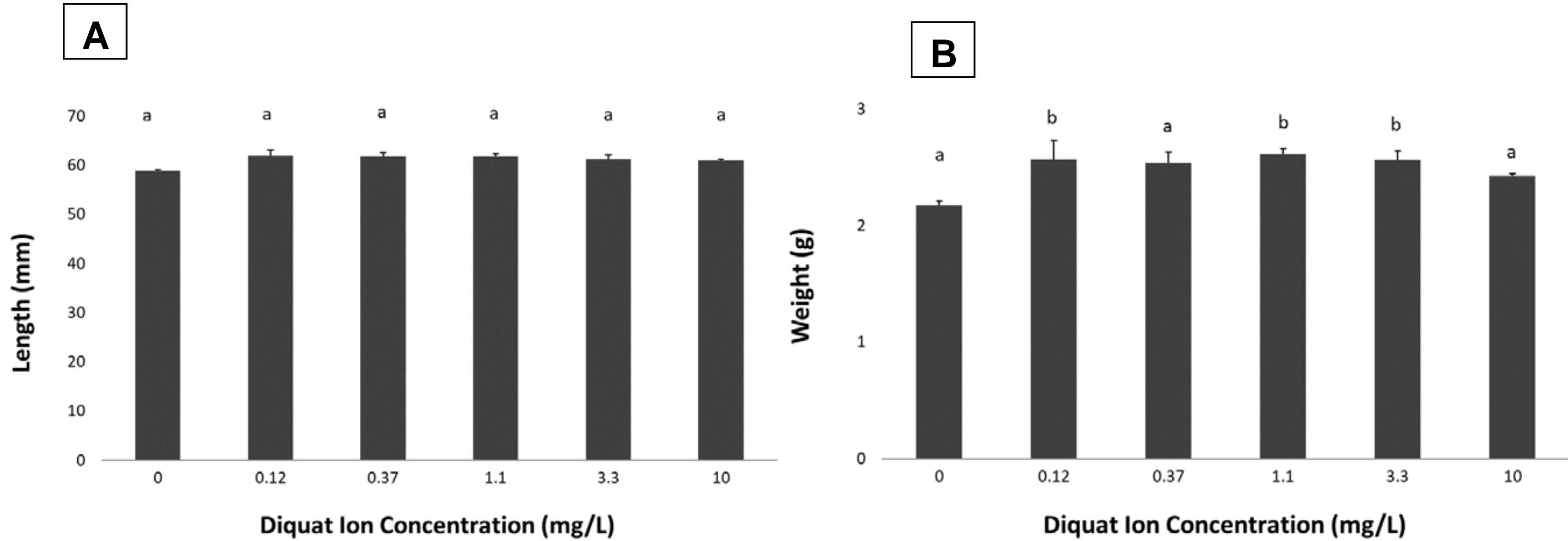


Figure 2.11 **The effects of juvenile life stage rainbow trout (66-86 d post hatch) following two 24 h pulse dose exposure to Reward® 14 d apart on A) length and B) weight (average of each replicate; total wet weight). Values presented are means ± standard error (n=4 tanks per treatment with 7 fish/tank). Different letters indicate significant difference between treatments (one way-analysis of variance followed by a Turkey's host-hoc, p<0.05). Exposure treatments were made up with Reward® Landscape and Aquatic Herbicide concentrate (240 g/L).**

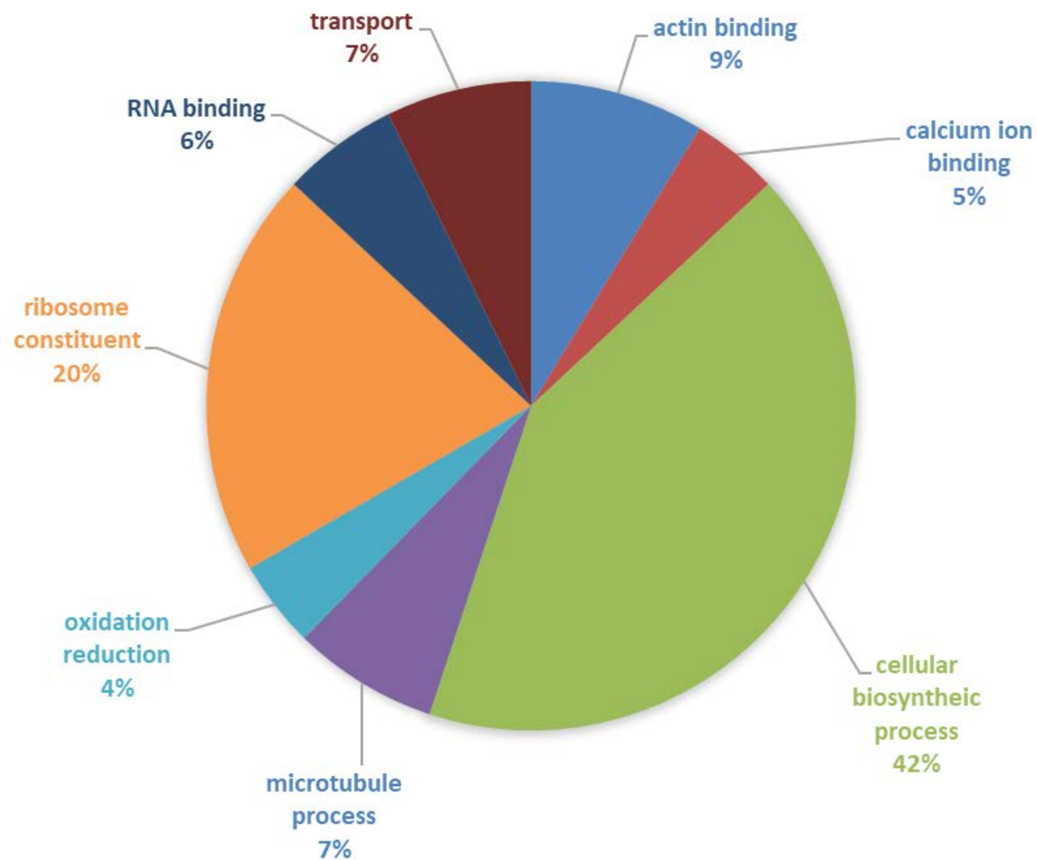


Figure 2.12 Percent distribution of Gene Ontology (GO) annotations of biological process and functions for identified hepatic proteins with significant expression changes from treatment fish vs controls for juvenile life stage rainbow trout (66-86 d post hatch). Exposures to juvenile life stage rainbow trout included two 24 h pulse exposures to Reward® Landscape and Aquatic Herbicide 14 d apart. If fewer than three proteins were identified for an annotation, then it is not displayed. Pie charts were constructed using Microsoft excel.

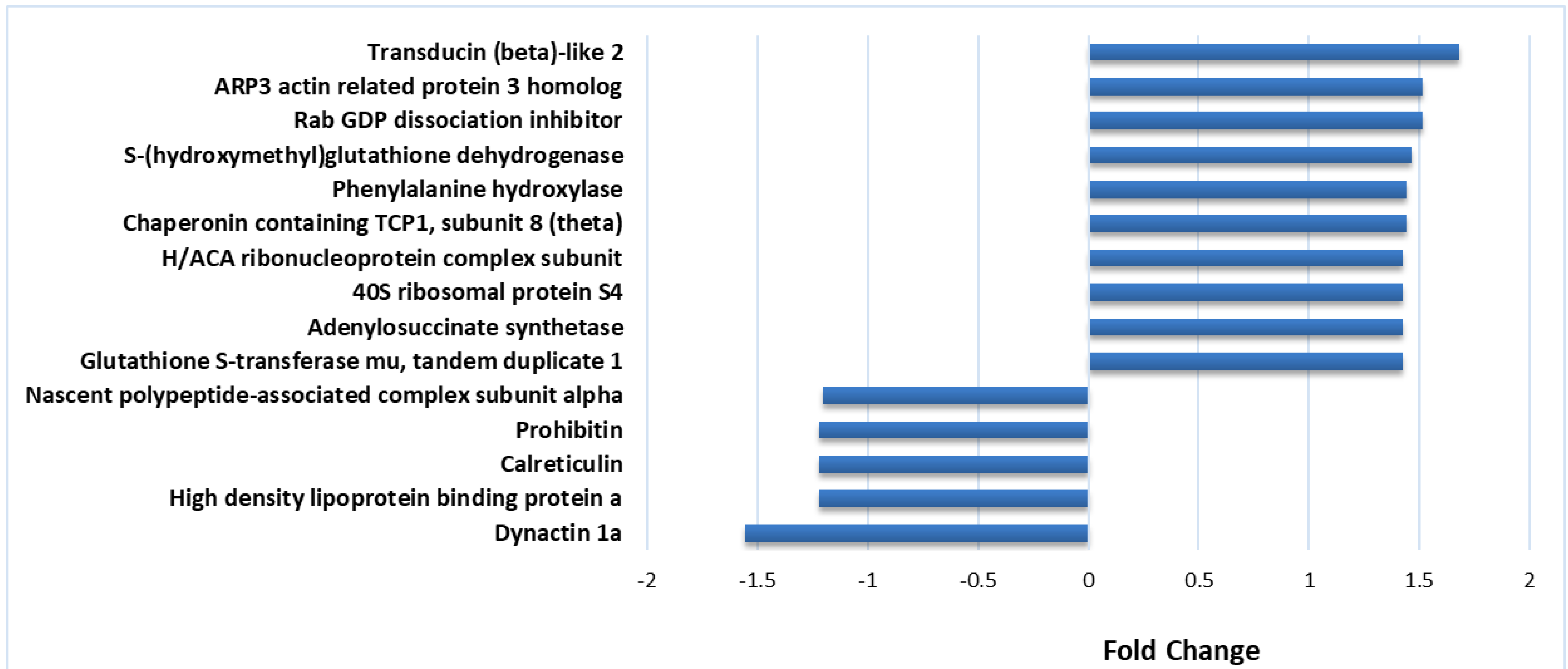


Figure 2.13 Variations in the expression of hepatic proteins after juvenile rainbow trout exposure to diquat dibromide using Reward® formulation. The 5 downregulated and 10 upregulated hepatic proteins with highest fold change are shown. Data were analyzed using Scaffold 4 Software and a Benjamini-Hochberg correction was applied to determine significance (p=<0.05).

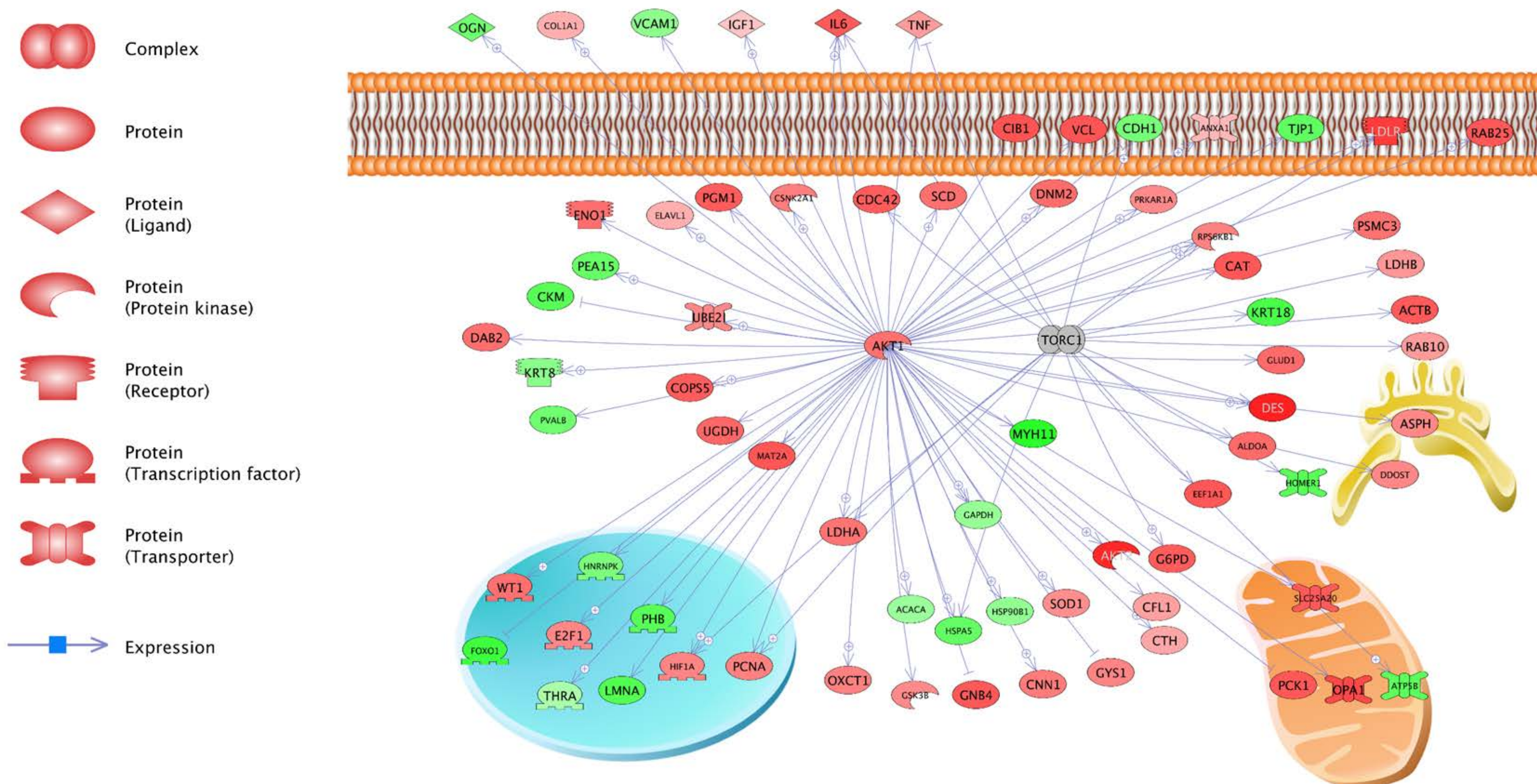


Figure 2.14 **Pathway studio sub-network enrichment analysis for differentially expressed proteins with the process of Atk/mTOR following two 24 h pulse dose exposures of Reward® Landscape and Aquatic Herbicide juvenile stage rainbow trout (66-86 d post hatch). Red indicates an increase in relative protein levels while green indicates a decrease in relative protein levels. Abbreviations follow that given in Supplemental Data.**

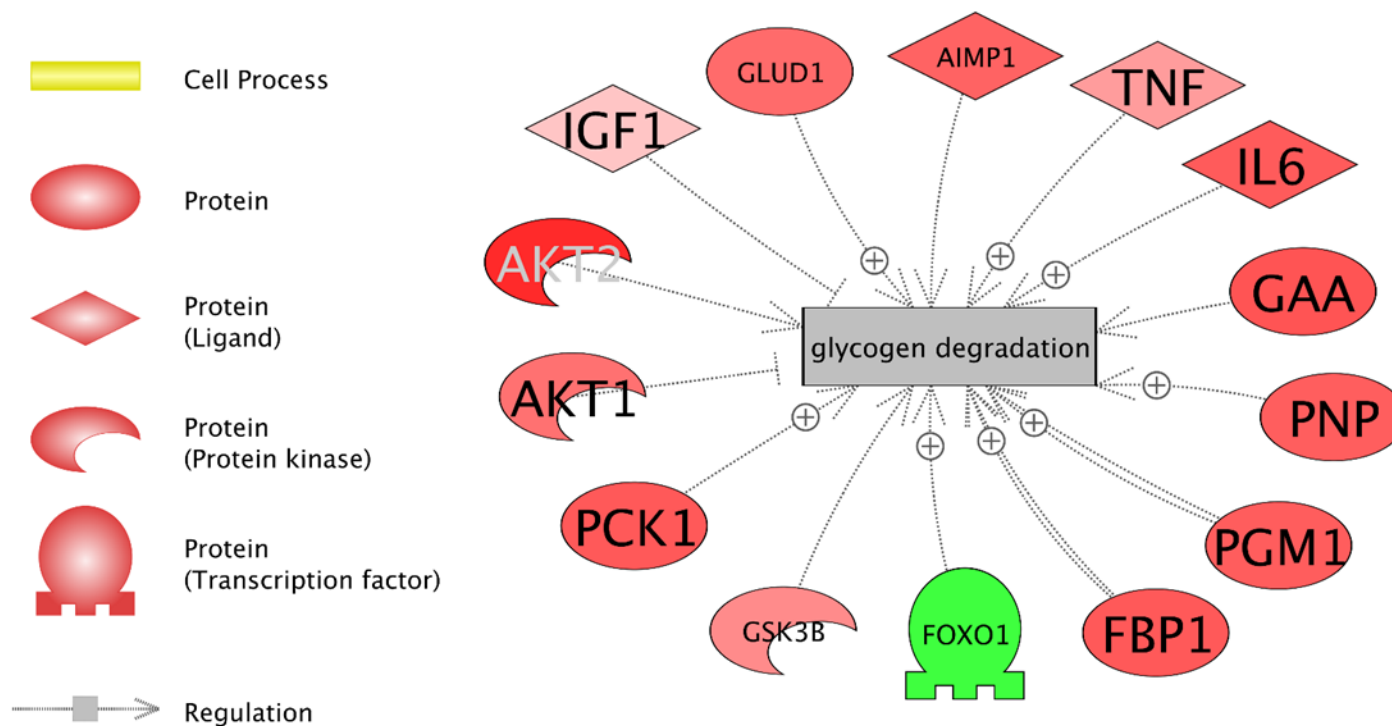


Figure 2.15. Pathway studio sub-network enrichment analysis for differentially expressed proteins with the process of glycogen degradation following two 24 h pulse dose exposures of Reward® Landscape and Aquatic Herbicide juvenile stage rainbow trout (66-86 d post hatch). Red indicates an increase in relative protein levels while green indicates a decrease in relative protein levels. Abbreviations follow that given in Supplemental Data.

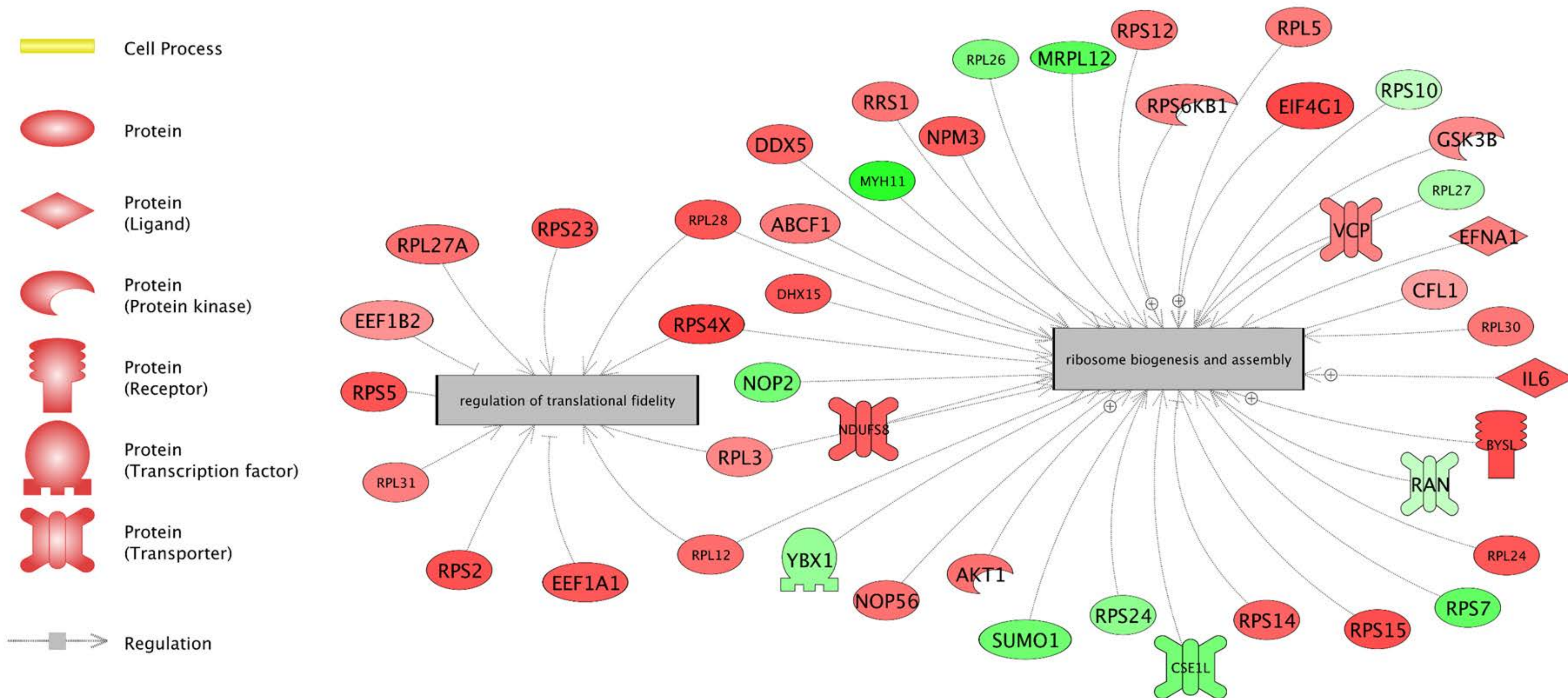


Figure 2.16. **Pathway studio sub-network enrichment analysis for differentially expressed proteins with the process of ribosome biogenesis and assembly and regulation of translational fidelity following two 24 h pulse dose exposures of Reward® Landscape and Aquatic Herbicide juvenile stage rainbow trout (66-86 d post hatch). Red indicates an increase in relative protein levels while green indicates a decrease in relative protein levels. Abbreviations follow that given in Supplemental Data.**

2.7. References

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Appendix A. Supplemental Information

Table A1 **Percent mortality of juvenile rainbow trout at various time points during a continuous 96 hour exposure to Reward®, a commercial formulation containing 240 g/L of diquat dibromide**

Exposure Duration (hr)	Diquat Dibromide Concentration (mg/L)														
	0 mg/L			6.5 mg/L			10 mg/L			15 mg/L			22 mg/L		
	Rep 1	Rep 2	Avg	Rep 1	Rep 2	Avg	Rep 1	Rep 2	Avg	Rep 1	Rep 2	Avg	Rep 1	Rep 2	Avg
24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
38	0	0	0	0	0	0	0	0	0	0	14	7	17	14	15
45	0	0	0	0	0	0	14	0	7	25	29	27	83	71	77
50	0	0	0	0	0	0	14	0	7	25	29	27	100	100	100
62	0	0	0	25	16	21	29	29	29	25	71	48	100	100	100
72	0	0	0	25	16	21	29	29	29	50	71	61	100	100	100
86	0	0	0	25	29	27	29	29	29	63	71	67	100	100	100
96	0	0	0	37	33	35	43	43	43	75	71	73	100	100	100

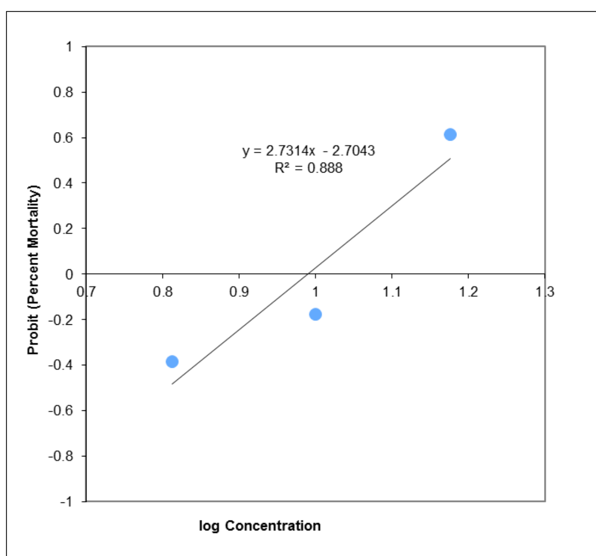
Note: Values represent the percent mortality of 2 replicate tanks of fish (7 fish/tank) of juvenile rainbow trout at various time points (hours). Fish age: 85 to 90 days post hatch

Table A2 **Percent mortality of juvenile rainbow trout at various time points during a continuous 96 hour exposure to Reward®, a commercial formulation containing 240 g/L of diquat dibromide**

Exposure Duration (hr)	Diquat Dibromide Concentration (mg/L)																	
	0 mg/L			0.37 mg/L			0.80 mg/L			1.8 mg/L			4.0 mg/L			8.7 mg/L		
	Rep 1	Rep 2	Avg	Rep 1	Rep 2	Avg	Rep 1	Rep 2	Avg	Rep 1	Rep 2	Avg	Rep 1	Rep 2	Avg	Rep 1	Rep 2	Avg
24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
48	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	14	7
72	0	0	0	0	0	0	14	0	7	0	0	0	0	0	0	0	43	22
96	0	0	0	0	0	0	14	0	7	0	0	0	0	0	0	29	57	43

Note: Values represent the percent mortality of 2 replicate tanks of fish (7 fish/tank) of juvenile rainbow trout at various time points (hours). Fish age: 56 to 60 days post hatch

Figure A3 **Probit anaysis graph showing percent mortality versus log concentration for juvenile rainbow trout exposed to Reward® during a continuous 96 hour exposure to Reward, a commercial formulation containing 240 g/L of diquat dibromide**



Note: Values represent the average percent mortality of 2 replicate tanks of fish (7 fish/tank) of juvenile rainbow trout age 85 to 90 days post hatch.

Table A4 **Hepatic protein expression changes for early life pre-feeding rainbow trout exposed to two 24 h pulse exposures to Reward®. The mean fold change of 3 biological replicates and significance value are shown.**

Accession Number	Protein Name	p value	Fold change
E6ZGG7_DICLA	Alpha-tropomyosin	0.008	3.5
H3CCD6_TETNG	Alpha-tropomyosin	0.008	3.5
H3DIM0_TETNG	Tropomyosin 2 (beta)	0.001	2.7
H3D1K5_TETNG	Parvalbumin 4	0.001	2.7
H3CAW8_TETNG	Desmin b	0.003	2.6
H3CMP8_TETNG	Creatine kinase muscle isoform 2	0.001	2.6
E6ZF31_DICLA	Creatine kinase brain isoform	< 0.0001	2.5
H3CBF5_TETNG	RAN binding protein 1	0.001	2.4
E6ZG88_DICLA	Chromosome 2 SCAF14695, whole genome shotgun sequence	< 0.0001	2.4
H3D1B9_TETNG	Desmin b	0.001	2.4
H3CI89_TETNG	Type II keratin E3	< 0.0001	2.4
H3D496_TETNG	DnaJ (Hsp40) homolog, subfamily C, member 8	0.001	2.3
H3BZL5_TETNG	Zgc:110239	0.001	2.3
Q4TBL7_TETNG	Tropomyosin 4a	0.002	2.3
H3CBE4_TETNG	ELAV-like protein	< 0.0001	2.3

Accession Number	Protein Name	p value	Fold change
H3CG97_TETNG	Calmodulin 1a	< 0.0001	2.2
H3C6H9_TETNG	TAF15 RNA polymerase II, TATA box binding protein (TBP)-associated factor	0.001	2.2
H3CXE8_TETNG	Splicing factor proline/glutamine-rich	0.004	2.2
H3DC26_TETNG	Heterogeneous nuclear ribonucleoprotein A0b	0.001	2.1
H3DPT3_TETNG	Chromosome undetermined SCAF11373, whole genome shotgun sequence	0.001	2.1
H3CLC5_TETNG	Chromosome undetermined SCAF7912, whole genome shotgun sequence	0.001	2.1
E6ZHI7_DICLA	Sorting nexin 12	0.005	2.1
Q4T6G9_TETNG	Interferon regulatory factor 2 binding protein 2	0.01	2.1
H3CZZ5_TETNG	Chromosome 12 SCAF14999, whole genome shotgun sequence	0.001	2.1
H3D6E6_TETNG	Chromosome 16 SCAF14974, whole genome shotgun sequence	< 0.0001	2.1
H3CP60_TETNG	Aminoacyl tRNA synthetase complex-interacting multifunctional protein 1	< 0.0001	2.1
H3D0E0_TETNG	Heterogeneous nuclear ribonucleoprotein A3	0.002	2.0
H3DPT3_TETNG	Chromosome undetermined SCAF14764, whole genome shotgun sequence	< 0.0001	2.0
E6ZHE6_DICLA	YTH N6-methyladenosine RNA binding protein 1	0.001	2.0
H3CIP0_TETNG	Eukaryotic translation initiation factor 4B	0.001	2.0
H3D2E7_TETNG	Metalloendopeptidase	0.001	2.0
H3DAU1_TETNG	Hnrp1	0.00048	2.0
H3D231_TETNG	Cofilin 1	0.001	2.0
H3D2E7_TETNG	H/ACA ribonucleoprotein complex subunit	0.001	2.0
H3CVX1_TETNG	N(alpha)-acetyltransferase 10, NatA catalytic subunit	< 0.0001	2.0
H3CCT7_TETNG	Chromosome 18 SCAF15124, whole genome shotgun sequence	0.001	2.0
Q4SFP9_TETNG	Lin-7 homolog C (C. elegans)	< 0.0001	2.0
H3CJ80_TETNG	Uncharacterized protein	0.001	1.9
H3CBA4_TETNG	KH-type splicing regulatory protein	0.001	1.9
Q4RMB7_TETNG	Mesencephalic astrocyte-derived neurotrophic factor	0.002	1.9
H3D0I6_TETNG	Branched chain keto acid dehydrogenase E1, alpha polypeptide	0.0006	1.9
H3CVU9_TETNG	Cathepsin D	0.001	1.9
H3CF53_TETNG	Tropomyosin 4	0.007	1.9
H3CUM2_TETNG	Dynactin 1a	0.006	1.9
Q4SNJ7_TETNG	Serine/arginine-rich splicing factor 6b	0.011	1.9
H3DBX3_TETNG	Sorting nexin 3	0.0004	1.9
Q4T3T1_TETNG	Calumenin b	0.001	1.9
Q4SMV9_TETNG	U1 small nuclear ribonucleoprotein C	0.004	1.9
Q4S1L4_TETNG	Small nuclear ribonucleoprotein D2 polypeptide	0.00079	1.9
H3D6B4_TETNG	Chromosome 1 SCAF14573, whole genome shotgun sequence	0.001	1.8
H3D8C5_TETNG	Small nuclear ribonucleoprotein-associated protein	0.001	1.8
H3CF52_TETNG	Adhesion regulating molecule 1	0.001	1.8
H3CU62_TETNG	Voltage-dependent anion channel 2	0.001	1.8
H3C6T3_TETNG	DnaJ (Hsp40) homolog, subfamily C, member 3b	0.005	1.8
H3CQN8_TETNG	LSM8 homolog, U6 small nuclear RNA associated	0.003	1.8

Accession Number	Protein Name	p value	Fold change
H3DBT4_TETNG	Chromosome 12 SCAF14996, whole genome shotgun sequence	0.004	1.8
H3DLI2_TETNG	Adenylate kinase 2, mitochondrial	< 0.0001	1.8
H3CCC3_TETNG	NudC nuclear distribution protein	0.001	1.8
H3CKM7_TETNG	Heterogeneous nuclear ribonucleoprotein H3	0.00025	1.8
H3CLY3_TETNG	Low molecular weight protein tyrosine phosphatase	0.001	1.8
H3CVD5_TETNG	Vasodilator-stimulated phosphoprotein b	0.001	1.8
Q4SKJ3_TETNG	V-crk avian sarcoma virus CT10 oncogene homolog-like	0.002	1.8
Q4SUD1_TETNG	NSFL1 (p97) cofactor (p47)	< 0.0001	1.8
H3D0A0_TETNG	Ribosomal protein S14	< 0.0001	1.8
H3DAS6_TETNG	Dynactin subunit 2	< 0.0001	1.8
H3CEK5_TETNG	Chromosome 15 SCAF14981, whole genome shotgun sequence	0.00034	1.8
H3DN72_TETNG	Cleavage stimulation factor, 3' pre-RNA, subunit 2	0.009	1.8
H3D8I7_TETNG	Nucleobindin 2a	< 0.0001	1.8
H3CVG4_TETNG	Chromosome undetermined SCAF14699, whole genome shotgun sequence	0.001	1.8
Q4S6E6_TETNG	NECAP endocytosis associated 2	0.001	1.8
H3CU89_TETNG	Protein LSM12 homolog A	0.002	1.8
H3CJ20_TETNG	Small ArfGAP 1	0.013	1.8
H3DAX4_TETNG	Chromosome undetermined SCAF14118, whole genome shotgun sequence	0.001	1.8
H3CD15_TETNG	Ribosomal protein L38	0.001	1.8
H3CJZ9_TETNG	Chromosome 18 SCAF14547, whole genome shotgun sequence	0.004	1.8
H3CJZ9_TETNG	Keratin 8	< 0.0001	1.8
Q4SFT9_TETNG	SNW domain containing 1	< 0.0001	1.8
H3CYY9_TETNG	Serine/arginine-rich splicing factor 1a	0.00012	1.7
H3DJD9_TETNG	BolA family member 3	0.001	1.7
H3CJQ4_TETNG	Voltage-dependent anion-selective channel protein 2	< 0.0001	1.7
H3CR51_TETNG	Biliverdin reductase B	0.001	1.7
H3BVV1_TETNG	Peptidylprolyl isomerase	0.001	1.7
H3D0G4_TETNG	DnaJ (Hsp40) homolog, subfamily A, member 2, like	0.004	1.7
H3CDL2_TETNG	Chromosome 11 SCAF10960, whole genome shotgun sequence	0.006	1.7
H3CD15_TETNG	Protein disulfide-isomerase	0.008	1.7
H3C768_TETNG	Uncharacterized protein	0.01	1.7
H3D420_TETNG	Insulin-like growth factor 2 mRNA binding protein 1	0.001	1.7
H3D552_TETNG	Poly(A) binding protein, nuclear 1	< 0.0001	1.7
H3DJU8_TETNG	Serine/arginine-rich splicing factor 5b	< 0.0001	1.7
A8QXK7_ANGAN	Mitochondrial ribosomal protein S31	0.001	1.7
H3CLX5_TETNG	Chromosome 14 SCAF14590, whole genome shotgun sequence	0.003	1.7

Accession Number	Protein Name	p value	Fold change
H3CLS0_TETNG	GTPase activating protein (SH3 domain) binding protein 2	0.00051	1.7
H3DFA2_TETNG	Myeloid/lymphoid or mixed-lineage leukemia; translocated to, 4a	0.00011	1.7
H3BZ80_TETNG	Serine/arginine-rich splicing factor 4	0.00014	1.7
H3D5R2_TETNG	Chromosome 15 SCAF14367, whole genome shotgun sequence	0.001	1.7
E6ZIV4_DICLA	Serine/arginine-rich splicing factor 9	0.002	1.7
H3CXA9_TETNG	DnaJ (Hsp40) homolog, subfamily A, member 2	0.004	1.7
H3BY52_TETNG	Nascent polypeptide-associated complex subunit alpha	< 0.0001	1.7
Q4STN8_TETNG	Splicing factor 3b, subunit 4	0.007	1.7
Q4S7T5_TETNG	Proteasome subunit alpha type	< 0.0001	1.7
H3CUQ7_TETNG	Polyadenylate-binding protein	0.00036	1.6
H3CU92_TETNG	Stress-induced phosphoprotein 1	< 0.0001	1.6
Q4SWL9_TETNG	Ankyrin repeat domain 40	0.004	1.6
M9T572_MEGAM	Chromosome undetermined SCAF10572, whole genome shotgun sequence	0.004	1.6
H3DI28_TETNG	Proteasome endopeptidase complex	< 0.0001	1.6
E6ZFZ0_DICLA	Myelin expression factor 2	0.01	1.6
H3DEV9_TETNG	RAD23 homolog B, nucleotide excision repair protein	0.003	1.6
Q4SSD9_TETNG	Putative RNA-binding protein Luc7-like 1	0.004	1.6
H3D0B3_TETNG	Polyadenylate-binding protein	< 0.0001	1.6
Q4RK67_TETNG	Methylmalonyl CoA epimerase	0.003	1.6
H3CLZ4_TETNG	Serine/arginine-rich splicing factor 1b	0.01	1.6
H3D659_TETNG	RNA binding motif protein 4.3	0.011	1.6
LDHB_FUNHE	Nucleoporin 62 like	0.001	1.6
H3DL71_TETNG	Nucleoside diphosphate kinase	0.009	1.6
H3CU75_TETNG	Triosephosphate isomerase	< 0.0001	1.6
H3C4S0_TETNG	Purine-rich element binding protein Ab	0.003	1.6
H3CCS4_TETNG	Voltage-dependent anion channel 1	< 0.0001	1.6
H3D7F2_TETNG	DnaJ (Hsp40) homolog, subfamily B, member 11	< 0.0001	1.6
H3CYT9_TETNG	Hydroxyprostaglandin dehydrogenase 15-(NAD)	0.001	1.6
Q4RMT6_TETNG	Tight junction protein 3	0.003	1.6
Q4RK97_TETNG	Filamin C, gamma b (actin binding protein 280)	0.004	1.6
A0A0U3HYC8_ORYME	Chromosome 4 SCAF14575, whole genome shotgun sequence	0.00082	1.5
H3CM65_TETNG	Aldehyde dehydrogenase 4 family, member A1	< 0.0001	1.5
H3CG64_TETNG	Voltage dependent anion channel 3	0.001	1.5
H3CNG9_TETNG	BCAS2, pre-mRNA processing factor	0.006	1.5
H3C2N6_TETNG	Chromosome 12 SCAF14999, whole genome shotgun sequence	0.01	1.5
H3DJB2_TETNG	Insulin-like growth factor 2 mRNA binding protein 3	0.002	1.5

Accession Number	Protein Name	p value	Fold change
M9T572_MEGAM	SUMO-conjugating enzyme	0.00038	1.5
H3CMD5_TETNG	Phosphoglycerate kinase	0.001	1.5
H3D556_TETNG	Phosphotriesterase-related protein	0.001	1.5
H3CIM0_TETNG	Chromosome 3 SCAF15050, whole genome shotgun sequence	0.001	1.5
H3DBJ2_TETNG	Ribosomal protein L31	0.001	1.5
H3DBJ2_TETNG	Suppression of tumorigenicity 13 (colon carcinoma) (Hsp70 interacting protein)	0.0026	1.5
H3C0G3_TETNG	dimethylarginine dimethylaminohydrolase 1	0.004	1.5
H3CFV4_TETNG	Ubiquilin 4	0.003	1.5
H3CIG8_TETNG	60S ribosomal protein L22	0.00038	1.5
H3D9X5_TETNG	RNA helicase p68b isoform m	0.00046	1.5
H3DD70_TETNG	Chromosome undetermined SCAF12162, whole genome shotgun sequence	0.00067	1.5
H3CJ82_TETNG	Pyruvate dehydrogenase E1 component subunit alpha	0.004	1.5
H3DN28_TETNG	Septin 2	0.00078	1.4
H3C488_TETNG	Eukaryotic translation initiation factor 3 subunit I	0.001	1.4
Q4RXG6_TETNG	Phosphate cytidylyltransferase 2, ethanolamine	0.001	1.4
E6ZG04_DICLA	Phosphate cytidylyltransferase 2, ethanolamine	0.001	1.4
H3DPV7_TETNG	40S ribosomal protein S10	0.001	1.4
H3DN67_TETNG	Glyceraldehyde-3-phosphate dehydrogenase	0.013	1.4
H3DJY6_TETNG	Capping protein (actin filament) muscle Z-line, beta	0.001	1.4
143B2_ONCMY	Small nuclear ribonucleoprotein polypeptide A'	0.0041	1.4
Q4RKM7_TETNG	SET nuclear proto-oncogene a	0.00015	1.4
Q4REX5_TETNG	Calreticulin	0.0004	1.4
M1VNS4_ONCMY	Dihydrolipoamide S-succinyltransferase	0.001	1.4
H3DAZ2_TETNG	Sorting nexin 1a	0.0028	1.4
H3DJI6_TETNG	Chromosome 21 SCAF7098, whole genome shotgun sequence	0.005	1.4
Q7T2K6_ONCMY	Fructose-bisphosphate aldolase	< 0.0001	1.4
E6ZH84_DICLA	RNA helicase p68b isoform m	0.001	1.4
H3D9F6_TETNG	Keratin 8	0.001	1.4
Q4SFM2_TETNG	26S protease regulatory subunit 8	< 0.0001	1.4
H3CCQ6_TETNG	Chromosome undetermined SCAF14784, whole genome shotgun sequence	0.001	1.4
H3CLM0_TETNG	Acyl-CoA dehydrogenase, C-4 to C-12 straight chain	0.00053	1.3
Q4S065_TETNG	26S protease regulatory subunit 8	0.001	1.3
A0A0N9H565_CYNSE	Protein disulfide-isomerase	0.001	1.3
H3CJL2_TETNG	Chromosome 7 SCAF14601, whole genome shotgun sequence	0.003	1.3
E6ZH84_DICLA	Ribosomal protein L12	0.003	1.3
H3D9P4_TETNG	CNDP dipeptidase 2 (metallopeptidase M20 family)	0.003	1.3
H3DDG9_TETNG	Carbonic anhydrase 2	0.013	1.3
H3C4W2_TETNG	E-cadherin	0.007	1.3

Accession Number	Protein Name	p value	Fold change
H3CSQ1_TETNG	CNDP dipeptidase 2 (metallopeptidase M20 family)	< 0.0001	1.3
Q4TBS0_TETNG	Peptidylprolyl isomerase	0.006	1.3
H3DH39_TETNG	Chromosome 18 SCAF15027, whole genome shotgun sequence	0.013	1.3
H3DAY9_TETNG	14-3-3 protein beta/alpha-2	< 0.0001	1.3
H3CMJ1_TETNG	Proteasome 26S subunit, ATPase 3	0.00073	1.3
H3D972_TETNG	NADH dehydrogenase (ubiquinone) Fe-S protein 3, (NADH-coenzyme Q reductase)	0.008	1.3
H3D972_TETNG	Isocitrate dehydrogenase [NAD] subunit, mitochondrial	0.002	1.3
E6ZEX1_DICLA	Calcineurin-like EF-hand protein 1	0.006	1.3
H3CRI0_TETNG	Chromosome 11 SCAF14979, whole genome shotgun sequence	0.01	1.2
H3CQY5_TETNG	Upf1 regulator of nonsense transcripts homolog (yeast)	0.006	1.2
H3CV53_TETNG	Heat shock protein 5	0.00014	1.2
E6ZGH1_DICLA	UDP-glucose 6-dehydrogenase	0.005	-1.2
A0A0N9H565_CYNSE	Phosphoenolpyruvate carboxykinase 2 (mitochondrial)	0.0062	-1.2
Q4SY70_TETNG	Proteasome 26S subunit, non-ATPase 2	0.011	-1.2
H3CXD2_TETNG	Valosin containing protein	< 0.0001	-1.2
H3CHM8_TETNG	Spectrin alpha 2	0.00057	-1.2
PTER_TETNG	Sarcosine dehydrogenase	0.003	-1.2
Q4RHA7_TETNG	Tubulin alpha chain	0.001	-1.2
H3DAK4_TETNG	Glutamic pyruvate transaminase (alanine aminotransferase) 2, like	0.004	-1.2
H3CHN4_TETNG	Glutamic pyruvate transaminase (alanine aminotransferase) 2, like	0.004	-1.2
H3DL55_TETNG	SEC22 homolog B, vesicle trafficking protein (gene/pseudogene)	0.00069	-1.3
H3CQC0_TETNG	Amidohydrolase domain containing 1	0.006	-1.3
E6ZGV8_DICLA	Heat shock protein 90 beta	0.006	-1.3
H3DK60_TETNG	ATP-binding cassette sub-family E member 1	0.001	-1.3
H3D7N3_TETNG	Adenosylhomocysteinase	0.003	-1.3
Q4SZ64_TETNG	Coatomer subunit alpha	0.004	-1.3
Q4RSR7_TETNG	Actin beta	0.006	-1.3
Q4SJL7_TETNG	ATP-binding cassette, sub-family B (MDR/TAP), member 11b	0.007	-1.3
H3CCF6_TETNG	Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1b	0.011	-1.3
H3D9C9_TETNG	Chromosome 18 SCAF15030, whole genome shotgun sequence	0.001	-1.3
H3D6N2_TETNG	Fructose-1,6-bisphosphatase 1b	0.00037	-1.3
H3CKW6_TETNG	C-terminal binding protein 1	0.0079	-1.3
H3CGB3_TETNG	Basic leucine zipper and W2 domains 1a	0.013	-1.3
H3DF87_TETNG	Pre-mRNA processing factor 8	0.0039	-1.3
H3CML7_TETNG	L-lactate dehydrogenase B chain	0.00017	-1.3
H3C3G2_TETNG	Phosphoglucomutase 1	0.001	-1.3
H3CBV8_TETNG	ATP-binding cassette, sub-family B (MDR/TAP), member 7	0.001	-1.3
Q4T8D3_TETNG	Aldehyde dehydrogenase 7 family, member A1	0.0034	-1.3
H3C428_TETNG	Uncharacterized protein	0.001	-1.3

Accession Number	Protein Name	p value	Fold change
H3CXA3_TETNG	Proteasome 26S subunit, non-ATPase 3	0.00097	-1.4
H3CJE6_TETNG	Staphylococcal nuclease and tudor domain containing 1	0.0051	-1.4
H3DPX4_TETNG	ARP3 actin related protein 3 homolog	0.01	-1.4
E6ZGT6_DICLA	ATP synthase subunit alpha	< 0.0001	-1.4
H3CUT0_TETNG	Myosin IB	0.0042	-1.4
H3CVW5_TETNG	26S proteasome non-ATPase regulatory subunit 6	0.008	-1.4
H3D8A5_TETNG	Acetoacetyl-CoA synthetase	0.012	-1.4
H3D517_TETNG	Chromosome undetermined SCAF13608, whole genome shotgun sequence	0.00078	-1.4
Q4T1J3_TETNG	Heat shock protein 90 beta	0.01	-1.4
H3CUT0_TETNG	Nicotinamide nucleotide transhydrogenase	0.0018	-1.4
H3CX13_TETNG	NAD(P) dependent steroid dehydrogenase-like	0.013	-1.4
H3DCN4_TETNG	Chromosome 18 SCAF14712, whole genome shotgun sequence	0.002	-1.4
H3CUT3_TETNG	Acetyltransferase component of pyruvate dehydrogenase complex	0.00034	-1.4
H3DP88_TETNG	40S ribosomal protein S24	0.00064	-1.4
H3C410_TETNG	Chromosome 1 SCAF14135, whole genome shotgun sequence	0.001	-1.4
Q4ZJF5_TETNG	Urocanate hydratase 1	0.0002	-1.4
H3BZ85_TETNG	Ras-related C3 botulinum toxin substrate 1b (rho family, small GTP binding protein Rac1)	0.002	-1.4
H3D2W2_TETNG	Glycyl-tRNA synthetase	0.0048	-1.4
H3BYI2_TETNG	Cullin-associated and neddylation-dissociated 1	0.00022	-1.5
H3D0E4_TETNG	Uncharacterized protein	0.001	-1.5
H3BYD7_TETNG	H2A histone family, member Y	0.013	-1.5
Q4SGV0_TETNG	Uncharacterized protein	0.00012	-1.5
H3D902_TETNG	Glucose-6-phosphate isomerase	0.001	-1.5
H3CIH4_TETNG	Phenylalanine hydroxylase	< 0.0001	-1.5
H3DG64_TETNG	Myosin, heavy chain 9b, non-muscle	0.00058	-1.5
H3DNS7_TETNG	Adaptor-related protein complex 2, alpha 1 subunit	0.0033	-1.5
H3C2C4_TETNG	Small nuclear ribonucleoprotein D1 polypeptide	0.011	-1.5
H3DPF8_TETNG	T-complex 1	< 0.0001	-1.5
Q4T0J8_TETNG	Ribosomal protein S15	0.0032	-1.5
H3CYG0_TETNG	Collagen, type VI, alpha 1	0.001	-1.5
H3DQX1_TETNG	Transmembrane p24 trafficking protein 10	0.003	-1.5
H3DFM2_TETNG	Oxoglutarate (alpha-ketoglutarate) dehydrogenase b (lipoamide)	0.001	-1.5
H3CVD8_TETNG	Oxoglutarate (alpha-ketoglutarate) dehydrogenase b (lipoamide)	0.001	-1.5
E6ZHH1_DICLA	Chaperonin containing TCP1, subunit 8 (theta)	0.001	-1.5
H3CWE6_TETNG	3-hydroxybutyrate dehydrogenase, type 2	0.002	-1.5
Q4STS2_TETNG	DNA helicase	0.004	-1.5
H3DAD0_TETNG	Uncharacterized protein	< 0.0001	-1.5
Q4SMI5_TETNG	Dihydropyrimidine dehydrogenase [NADP(+)]	0.00029	-1.5

Accession Number	Protein Name	p value	Fold change
H3CJL2_TETNG	Adenosine monophosphate deaminase 2a	0.001	-1.5
Q4S9B4_TETNG	Adaptor-related protein complex 2, alpha 1 subunit	0.0019	-1.5
H3DAZ7_TETNG	Chromosome 10 SCAF14728, whole genome shotgun sequence	0.005	-1.5
E6ZHW6_DICLA	O-linked N-acetylglucosamine (GlcNAc) transferase, tandem duplicate 1	0.01	-1.5
H3DPX1_TETNG	Chaperonin containing TCP1, subunit 7 (eta)	0.004	-1.5
Q4RWP9_TETNG	Coatomer subunit beta	0.008	-1.5
H3DHH9_TETNG	Uncharacterized protein	0.0004	-1.6
H3CVH0_TETNG	V-type proton ATPase subunit	0.001	-1.6
H3D8E9_TETNG	60S acidic ribosomal protein P0	0.0008	-1.6
U5JDR3_SCOMX	Protein kinase, AMP-activated, gamma 1 non-catalytic subunit	0.001	-1.6
H3C0M1_TETNG	Histone H4	0.00019	-1.6
H3CKX2_TETNG	Chromosome undetermined SCAF13964, whole genome shotgun sequence	0.004	-1.6
H3CAQ1_TETNG	N-ethylmaleimide-sensitive factor b	0.003	-1.6
H3CQ87_TETNG	Karyopherin (importin) beta 1	0.0063	-1.6
E6ZF86_DICLA	Lysine--tRNA ligase	0.009	-1.6
H3CJA8_TETNG	Uncharacterized protein	0.002	-1.6
H3CB08_TETNG	Leucyl-tRNA synthetase b	0.001	-1.6
Q4RTW8_TETNG	Adaptor-related protein complex 2, sigma 1 subunit	0.001	-1.6
H3D185_TETNG	Coiled-coil domain containing 47	0.0057	-1.6
Q4SJN9_TETNG	Karyopherin (importin) beta 3	0.00019	-1.6
H3CBD8_TETNG	Heat shock protein 4b	0.005	-1.6
H3D4L4_TETNG	UDP-glucose glycoprotein glucosyltransferase 1	0.007	-1.6
H3CT85_TETNG	Ribosomal protein L5	0.001	-1.6
H3D639_TETNG	Signal recognition particle subunit SRP72	0.013	-1.7
H3D2E9_TETNG	Annexin	0.011	-1.7
E6ZEW7_DICLA	Annexin	0.001	-1.7
H3D410_TETNG	Nucleolar GTP-binding protein 1	0.0018	-1.7
Q4REY8_TETNG	Coatomer subunit gamma	0.0039	-1.7
H3CLQ0_TETNG	Chromosome undetermined SCAF9929, whole genome shotgun sequence	0.004	-1.7
H3C243_TETNG	Chromosome 6 SCAF14544, whole genome shotgun sequence	0.012	-1.7
Q4RRE6_TETNG	Chromosome 10 SCAF15019, whole genome shotgun sequence	0.00024	-1.7
CATD_CHIHA	Coatomer subunit beta'	0.00037	-1.7
H3BWT4_TETNG	Solute carrier family 27 (fatty acid transporter), member 2a	0.001	-1.7
H3D983_TETNG	NADH dehydrogenase (ubiquinone) Fe-S protein 1	0.002	-1.7
H3CI62_TETNG	Ribosomal protein S18	0.001	-1.7
H3CPQ0_TETNG	Ribosomal protein L9	0.003	-1.7
Q4REB5_TETNG	Uncharacterized protein	0.001	-1.8
H3BW32_TETNG	Alpha-1,4 glucan phosphorylase	0.012	-1.8
H3D662_TETNG	COP9 signalosome subunit 2	0.0015	-1.8
H3DCA4_TETNG	Ubiquitin specific peptidase 7 (herpes virus-associated)	0.00076	-1.8
H3DHD8_TETNG	Clathrin heavy chain	< 0.0001	-1.8
A8DSX7_HAPBU	Actin-related protein 2/3 complex subunit	0.00022	-1.8

Accession Number	Protein Name	p value	Fold change
H3CEB0_TETNG	Carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase	0.004	-1.8
E6ZFG9_DICLA	Clathrin heavy chain	< 0.0001	-1.8
H3CZR0_TETNG	Ribosomal protein L19	0.00027	-1.9
H3C4Z3_TETNG	Alpha-1,4 glucan phosphorylase	0.001	-1.9
Q4S1X2_TETNG	Eukaryotic translation initiation factor 3 subunit L	0.00017	-1.9
Q4RSP0_TETNG	Glycogen [starch] synthase	0.001	-1.9
Q4RZ13_TETNG	Acidic (leucine-rich) nuclear phosphoprotein 32 family, member B	0.003	-1.9
H3DKZ5_TETNG	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit	0.0002	-1.9
H3DGW6_TETNG	Chromosome undetermined SCAF8763, whole genome shotgun sequence	0.002	-1.9
Q4T824_TETNG	GTP-binding protein SAR1b	0.0053	-2.0
H3DLJ4_TETNG	Alpha-1,4 glucan phosphorylase	0.00017	-2.0
Q4T029_TETNG	Aminopeptidase	< 0.0001	-2.0
H3CPM1_TETNG	Ubiquitin-like modifier activating enzyme 1	0.001	-2.0
H3D188_TETNG	Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5	< 0.0001	-2.1
H3CEJ4_TETNG	Valyl-tRNA synthetase	0.001	-2.2
H3CQN4_TETNG	T-complex protein 1 subunit gamma	0.00016	-2.2
H3DRE0_TETNG	Glutamine--fructose-6-phosphate transaminase 1	0.001	-2.2
H3C1E4_TETNG	Adaptor-related protein complex 1, gamma 1 subunit	0.001	-2.3
H3BZ06_TETNG	Developmentally regulated GTP binding protein 2	0.002	-2.3
H3DJM5_TETNG	DEAH (Asp-Glu-Ala-His) box helicase 15	< 0.0001	-2.3
E6ZFH2_DICLA	Aconitate hydratase	0.00038	-2.4
H3DIE5_TETNG	Signal peptidase complex catalytic subunit SEC11	0.001	-2.4
H3DH78_TETNG	Cell division control protein 42 homolog	0.003	-2.4
Q4S9T8_TETNG	myosin light chain kinase, smooth muscle-like isoform X3	0.008	-2.6
Q7T1J1_CHAAC	Glycerol-3-phosphate dehydrogenase [NAD(+)]	0.0074	-3.0
Q7T1J2_CHAAC	Non-specific serine/threonine protein kinase	0.007	-3.2
H3DIE5_TETNG	X-prolyl aminopeptidase (aminopeptidase P) 1, soluble	0.003	-4.5
H3D5S1_TETNG	Calcium-transporting ATPase	0.002	-4.7
H3CHP2_TETNG	Serine/threonine-protein phosphatase	0.001	-5.4
H3BYP9_TETNG	Cell division cycle 42, like	0.007	-10.3
H3BYP9_TETNG	Pantothenate kinase 4	0.003	-13.6

Table A5 Hepatic protein expression changes for juvenile feeding rainbow trout exposed to two 24 h pulse exposures to Reward®. The mean fold change of 4 biological replicates and significance value are shown.

Accession Number	Protein Name	P value	Fold Change
H3CQD0_TETNG	Transducin (beta)-like 2	0.001	1.7
Q4S684_TETNG	Rab GDP dissociation inhibitor	0.002	1.5
Q4RK67_TETNG	ARP3 actin related protein 3 homolog	0.002	1.5
H3D661_TETNG	S-(hydroxymethyl)glutathione dehydrogenase	0.001	1.5
Q4SFT9_TETNG	Chaperonin containing TCP1, subunit 8 (theta)	0.001	1.4
H3D552_TETNG	Phenylalanine hydroxylase	0.003	1.4
H3DCF7_TETNG	Glutathione S-transferase mu, tandem duplicate 1	0.00066	1.4
H3DJA4_TETNG	Adenylosuccinate synthetase	0.003	1.4
H3CB17_TETNG	40S ribosomal protein S4	< 0.0001	1.4
H3D662_TETNG	H/ACA ribonucleoprotein complex subunit	< 0.0001	1.4
H3CUM2_TETNG	Annexin	0.00024	1.4
H3CVD5_TETNG	Lysine--tRNA ligase	0.002	1.4
H3CTK6_TETNG	Regulator of microtubule dynamics 1	< 0.0001	1.4
H3CAD0_TETNG	Succinate--CoA ligase [ADP/GDP-forming] subunit alpha, mitochondrial	0.00035	1.4
Q4S065_TETNG	Chromosome undetermined SCAF14784, whole genome shotgun sequence	< 0.0001	1.4
E6ZFZ0_DICLA	26S proteasome non-ATPase regulatory subunit 6	< 0.0001	1.4
H3DDZ4_TETNG	Hydroxyacid oxidase 2 (long chain)	0.001	1.4
H3D293_TETNG	Ribosomal protein S11	< 0.0001	1.3
H3CWT5_TETNG	ARP2 actin related protein 2 homolog	0.0003	1.3
H3D8I7_TETNG	Chaperonin containing TCP1, subunit 7 (eta)	0.004	1.3
H3D2E9_TETNG	Small nuclear ribonucleoprotein D2 polypeptide	< 0.0001	1.3
H3CUZ2_TETNG	Trafficking protein particle complex subunit	0.003	1.3
H3DKX6_TETNG	Ribosomal protein S2	0.0004	1.3
H3DL55_TETNG	dimethylarginine dimethylaminohydrolase 1	0.0019	1.3
H3CK52_TETNG	Hypoxanthine phosphoribosyltransferase 1	< 0.0001	1.3
H3D535_TETNG	Ribosomal protein S16	< 0.0001	1.3
H3CG97_TETNG	T-complex protein 1 subunit gamma	0.004	1.3
A0P9L5_ONCMY	ribosomal protein S23	0.00024	1.3
H3C4B5_TETNG	Ribosomal protein S5	0.00034	1.3
CISY_TETNG	Citrate synthase, mitochondrial	0.00036	1.3
Q4S7D6_TETNG	Calcium and integrin binding 1 (calmyrin)	0.003	1.3
RS3A_TETNG	40S ribosomal protein S3a	< 0.0001	1.3
H3D0A0_TETNG	60S acidic ribosomal protein P0	< 0.0001	1.3
H3C0A1_TETNG	Ribosomal protein L28	0.00011	1.3

Accession Number	Protein Name	P value	Fold Change
H3C4S0_TETNG	Aldehyde dehydrogenase 7 family, member A1	0.00041	1.3
H3D6H4_TETNG	FACT complex subunit SSRP1	0.00066	1.3
H3CCP7_TETNG	Eukaryotic translation termination factor 1a	0.00076	1.3
Q4SW80_TETNG	Ras-related GTP-binding Ca	0.001	1.3
H3CF68_TETNG	Increased sodium tolerance 1 homolog (yeast)	0.003	1.3
Q8JFQ8_PLEAT	Carbonyl reductase	0.004	1.3
H3D7F2_TETNG	Fructose-1,6-bisphosphatase 1b	0.00036	1.3
H3D8R5_TETNG	Ribosomal protein L24	0.00086	1.3
Q5XW25_OPLFA	Catalase	< 0.0001	1.3
H3CMG9_TETNG	Propionyl CoA carboxylase, beta polypeptide	0.00023	1.2
H3CM65_TETNG	Adenosylhomocysteinase	0.00075	1.2
H3CI62_TETNG	Mesencephalic astrocyte-derived neurotrophic factor	0.001	1.2
Q4RYW0_TETNG	Calcium binding protein 39	0.0019	1.2
H3CJG9_TETNG	Solute carrier family 25 (mitochondrial carrier; dicarboxylate transporter), member 10	0.002	1.2
H3CHE3_TETNG	Interleukin enhancer binding factor 2	< 0.0001	1.2
H3CA69_TETNG	Signal recognition particle 54 kDa protein	0.00033	1.2
H3DL71_TETNG	Phosphoglucomutase 1	0.001	1.2
H3CLS5_TETNG	Flotillin 2b	0.002	1.2
H3C410_TETNG	Serine/arginine-rich splicing factor 4	0.0027	1.2
H3DF09_TETNG	Histidine ammonia-lyase	0.00047	1.2
Q4SDS8_TETNG	Chromosome 1 SCAF14632, whole genome shotgun sequence	0.00085	1.2
Q4S739_TETNG	Chromosome 14 SCAF14723, whole genome shotgun sequence	0.0037	1.2
H3CAG3_TETNG	Ribosomal protein S17	0.00024	1.2
H3DJI6_TETNG	CNDP dipeptidase 2 (metallopeptidase M20 family)	0.00046	1.2
Q4TAL3_TETNG	Chromosome undetermined SCAF7287, whole genome shotgun sequence	0.001	1.2
H3DKZ5_TETNG	Aminoacyl tRNA synthetase complex-interacting multifunctional protein 1	0.002	1.2
Q4SMC4_TETNG	Arp2/3 complex 34 kDa subunit	0.004	1.2
H3CR86_TETNG	Proteasome subunit beta type	0.00022	1.2
H3D0V6_TETNG	Ribosomal protein L35a	0.002	1.2
H3CPX6_TETNG	Ribosomal protein L7a	0.00013	1.2
H3D420_TETNG	T-complex 1	0.001	1.2
H3CQN8_TETNG	Adaptor-related protein complex 2, sigma 1 subunit	0.00016	1.2
Q4S9B4_TETNG	Chromosome undetermined SCAF14699, whole genome shotgun sequence	0.00044	1.2
H3CRF3_TETNG	Flotillin 1b	0.00068	1.2
H3D9P4_TETNG	Fructose-bisphosphate aldolase	0.001	1.2

Accession Number	Protein Name	P value	Fold Change
Q4SDJ7_TETNG	Chromosome 18 SCAF14637, whole genome shotgun sequence	0.001	1.2
H3CJ80_TETNG	Ribosomal protein L9	0.002	1.1
H3CCF6_TETNG	Aldehyde dehydrogenase 4 family, member A1	0.002	1.1
H3D1U3_TETNG	Heat shock 60 protein 1	0.002	1.1
E6ZJ78_DICLA	Poly(U)-binding-splicing factor PUF60	0.002	1.1
H3CAQ1_TETNG	NSFL1 (p97) cofactor (p47)	0.002	-1.1
E6ZF86_DICLA	Dynactin subunit 2	0.00075	-1.1
CATD_CHIHA	Cathepsin D	0.004	-1.2
H3BYI2_TETNG	Nascent polypeptide-associated complex subunit alpha	0.00024	-1.2
H3CEY2_TETNG	High density lipoprotein binding protein a	0.00019	-1.2
H3DDG9_TETNG	Calreticulin	0.0018	-1.2
H3CB35_TETNG	Prohibitin	0.002	-1.2
H3CLQ0_TETNG	Dynactin 1a	0.00048	-1.3
H3D2H2_TETNG	Dynactin 1a	0.001	-1.4
H3CNJ1_TETNG	Dynactin 1a	0.002	-1.6

Table A6 Sub-network enrichment analysis pathway abbreviations.

Code	Protein Name
sterol regulatory element binding protein	
LRP1	LDL receptor related protein 1
PCK1	phosphoenolpyruvate carboxykinase 1
NPC1	Niemann-Pick disease, type C1
SLC25A1	solute carrier family 25 member 1
ABCB4	ATP binding cassette subfamily B member 4
ABCA1	ATP binding cassette subfamily A member 1
HNF4A	hepatocyte nuclear factor 4 alpha
PCYT1A	phosphate cytidylyltransferase 1, choline, alpha
MAT1A	methionine adenosyltransferase 1A
PKLR	pyruvate kinase, liver and RBC
ABCC2	ATP binding cassette subfamily C member 2
GNAI2	G protein subunit alpha i2
IL6	interleukin 6
DDAH1	dimethylarginine dimethylaminohydrolase 1
MTTP	microsomal triglyceride transfer protein
G6PD	glucose-6-phosphate dehydrogenase
IDH1	isocitrate dehydrogenase 1 (NADP+)
ACSS2	acyl-CoA synthetase short-chain family member 2
CASP3	
LMNA	lamin A/C
TJP1	tight junction protein 1
AIMP1	aminoacyl tRNA synthetase complex-interacting multifunctional protein 1
DES	desmin
AKT1	v-akt murine thymoma viral oncogene homolog 1
CASP3	caspase 3
MAP1A	microtubule associated protein 1A
MLLT4	myeloid/lymphoid or mixed-lineage leukemia; translocated to, 4
CYP1A1	cytochrome P450 family 1 subfamily A member 1
MME	membrane metallo-endopeptidase
PARP1	poly(ADP-ribose) polymerase 1
CALM3	calmodulin 3 (phosphorylase kinase, delta)
CDH1	cadherin 1
PCNA	proliferating cell nuclear antigen
SPTB	spectrin beta, erythrocytic
retinoid-X receptor	
ABCB11	ATP binding cassette subfamily B member 11

Code	Protein Name
ANXA5	annexin A5
PCK1	phosphoenolpyruvate carboxykinase 1
SLC27A2	solute carrier family 27 member 2
ABCA1	ATP binding cassette subfamily A member 1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
ABCG2	ATP binding cassette subfamily G member 2 (Junior blood group)
PRDX3	peroxiredoxin 3
PCYT1A	phosphate cytidylyltransferase 1, choline, alpha
PRDX1	peroxiredoxin 1
VTLGL1	vitellogenin-like 1
FABP6	fatty acid binding protein 6
LDHA	lactate dehydrogenase A
ENO1	enolase 1
FLOT2	flotillin 2
APOE	apolipoprotein E
SNCA	synuclein alpha
EGFR	epidermal growth factor receptor
CPT1A	carnitine palmitoyltransferase 1A
HSPB1	heat shock protein family B (small) member 1
ABCB4	ATP binding cassette subfamily B member 4
UCP1	uncoupling protein 1 (mitochondrial, proton carrier)
FLOT1	flotillin 1
CSNK2B	casein kinase 2 beta
CA2	carbonic anhydrase 2
TNF	tumor necrosis factor
HNF4A	hepatocyte nuclear factor 4 alpha
RBP1	retinol binding protein 1
PRDX2	peroxiredoxin 2
CYP1A1	cytochrome P450 family 1 subfamily A member 1
ABCC2	ATP binding cassette subfamily C member 2
IL6	interleukin 6
DDAH1	dimethylarginine dimethylaminohydrolase 1
MTTP	microsomal triglyceride transfer protein
RBP2	retinol binding protein 2
ribosome biogenesis and assembly	
RPL3	ribosomal protein L3
EIF4G1	eukaryotic translation initiation factor 4 gamma 1
RPL5	ribosomal protein L5
RPS12	ribosomal protein S12
RPS7	ribosomal protein S7

Code	Protein Name
BYSL	bystin like
RAN	RAN, member RAS oncogene family
RPS14	ribosomal protein S14
RPS15	ribosomal protein S15
RPL12	ribosomal protein L12
RPS10	ribosomal protein S10
DDX5	DEAD-box helicase 5
ABCF1	ATP binding cassette subfamily F member 1
CSE1L	chromosome segregation 1 like
NDUFS8	NADH:ubiquinone oxidoreductase core subunit S8
RPL26	ribosomal protein L26
RPL28	ribosomal protein L28
VCP	valosin containing protein
RRS1	ribosome biogenesis regulator homolog
DHX15	DEAH-box helicase 15
EFNA1	ephrin A1
RPS24	ribosomal protein S24
RPL27	ribosomal protein L27
RPL24	ribosomal protein L24
RPL30	ribosomal protein L30
YBX1	Y-box binding protein 1
SUMO1	small ubiquitin-like modifier 1
NOP2	NOP2 nucleolar protein
AKT1	v-akt murine thymoma viral oncogene homolog 1
GSK3B	glycogen synthase kinase 3 beta
MRPL12	mitochondrial ribosomal protein L12
CFL1	cofilin 1
NPM3	nucleophosmin/nucleoplasmin 3
NOP56	NOP56 ribonucleoprotein
IL6	interleukin 6
RPS6KB1	ribosomal protein S6 kinase B1
RPS4X	ribosomal protein S4, X-linked
MYH11	myosin, heavy chain 11, smooth muscle
mRNA metabolism	
SRSF1	serine/arginine-rich splicing factor 1
HNRNPA1	heterogeneous nuclear ribonucleoprotein A1
SRSF2	serine/arginine-rich splicing factor 2
HNRNPK	heterogeneous nuclear ribonucleoprotein K
COL1A1	collagen type I alpha 1
ACTN4	actinin alpha 4

Code	Protein Name
ELAVL1	ELAV like RNA binding protein 1
FUS	FUS RNA binding protein
USP39	ubiquitin specific peptidase 39
HNRNPA0	heterogeneous nuclear ribonucleoprotein A0
SEPT9	septin 9
SND1	staphylococcal nuclease and tudor domain containing 1
CYCS	cytochrome c, somatic
MAGOH	mago homolog, exon junction complex core component
RBM4	RNA binding motif protein 4
HSPA5	heat shock protein family A (Hsp70) member 5
HDLBP	high density lipoprotein binding protein
AKT1	v-akt murine thymoma viral oncogene homolog 1
PSMC5	proteasome 26S subunit, ATPase 5
PABPN1	poly(A) binding protein nuclear 1
TIA1	TIA1 cytotoxic granule-associated RNA binding protein
G3BP1	G3BP stress granule assembly factor 1
KHDRBS1	KH domain containing, RNA binding, signal transduction associated 1
PABPC1	poly(A) binding protein cytoplasmic 1
KHSRP	KH-type splicing regulatory protein
PCBP2	poly(rC) binding protein 2
SRSF7	serine/arginine-rich splicing factor 7
protein splicing	
SRSF11	serine/arginine-rich splicing factor 11
SRSF1	serine/arginine-rich splicing factor 1
SRSF5	serine/arginine-rich splicing factor 5
RAF1	Raf-1 proto-oncogene, serine/threonine kinase
HNRNPA1	heterogeneous nuclear ribonucleoprotein A1
SRSF2	serine/arginine-rich splicing factor 2
SRSF4	serine/arginine-rich splicing factor 4
RBM4	RNA binding motif protein 4
SRSF6	serine/arginine-rich splicing factor 6
TRA2B	transformer 2 beta homolog (Drosophila)
SNW1	SNW domain containing 1
ATP6V1A	ATPase H+ transporting V1 subunit A
SRSF9	serine/arginine-rich splicing factor 9
LONP1	lon peptidase 1, mitochondrial
SRSF7	serine/arginine-rich splicing factor 7