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Research article

Differential protein abundance in the regenerating Caribbean corallimorpharian *Ricordea florida*

Ryan A. Horricks[1,2], Zachary Millar[1], Kyra Sykes[1], John S. Lumsden[1,2]

[1]*Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph ON, N1G 2W1, Canada.*
[2]*Department of Pathobiology, School of Veterinary Medicine, St. George's University, True Blue, Grenada, West Indies.*

Correspondence: John S. Lumsden, email; jsl@uoguelph.ca

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Abstract

One remarkable ability of cnidarians is their capacity to completely regenerate damaged tissue. Studies on tissue regeneration in corals in a natural setting can be challenging due to environmental variation, time and obtaining permits. Therefore, the proteomics of coral tissue regeneration in the captive corallimorpharian *Ricordea florida* were examined. Corallimorpharians regenerate quickly and provide copious tissue for analysis. A more detailed understanding of fragmentation and regeneration in corallimorpharians is important because sexual reproduction has not been recorded in captivity. Instead, captive population increases rely exclusively on asexual reproduction, or fragmentation, for propagation. To examine temporal protein variability during regeneration, 36 *R. florida* polyps were bisected across the mouth and allowed to regenerate. Regenerating whole-animal samples were collected at 6, 12 and 24 hr, as well as 2, 4 and 14 d following bisection. Tissue samples were labelled using isobaric tandem mass tags and liquid chromatography-mass spectrometry was used to quantify protein abundance as the polyps regenerated. One hundred proteins were significantly differentially abundant over the course of the 14-day experimental period. Cytochrome c, a known trigger of cell death in mammals and critical to energy metabolism in corals, was more abundant during regeneration. Identification of protein biomarkers common to regenerating corals will allow development of tools to track protein expression during stressful events. Improved understanding of regeneration can contribute to improved corallimorpharian propagation in captivity, reducing demand from the aquarium industry for wild-harvested individuals.

Introduction

The persistence of coral on reefs faced with numerous anthropogenic threats partly depends on their ability to regenerate damaged tissue. Wound healing in mammalian and reptilian models can result in scarring (Bely and Nyberg 2010; Henry and Hart 2005) whereas tissue regeneration recapitulates damaged tissue without scarring (Bely and Nyberg 2010). This phenomenon is common in many anthozoans and has been documented in *Aiptasia* spp. (Baumgarten et al. 2015) and *Hydra vulgaris* (Zacharias et al. 2004).

Closely related to scleractinian corals (Lin et al. 2017), corallimorpharians are commonly found in tropical waters (Chadwick-Furman and Spiegel 2000) as either individual polyps (den Hartog 1980) or in large colonial aggregations (Muhando et al. 2002) and have symbiotic zooxanthellae (Lin et al. 2017). The order contains four families and approximately twenty-four species (Daly et al. 2007; Torres-Pratts et al. 2011). Despite their close phylogenetic relationship, corallimorpharians lack the defining calcareous skeleton of scleractinian corals but share the ability to regenerate damaged tissue (Vroom 2016). Regeneration following bisection in the corallimorpharian *Rhodactis* sp. is characterised by tissue extension, sealing, reorganisation and differentiation, and is often completed within one week (Vroom 2016).

Corallimorpharians, including *Ricordea florida*, are heavily harvested from the wild for the aquarium industry (Torres-Pratts et al. 2011), however losses in transit and captivity

are high (Livengood and Chapman 2007). Coral aquaculture can alleviate some of the demand (Barton et al. 2017), hence suitable techniques for propagation are needed for each group of corals. Corals in captivity that are fragmented too frequently have poor survival (Millar personal communication). It is possible that such coral fragments can undergo decline if energy budgets are exceeded, but the reasons for this are not currently understood. Storm-fragmented *A. palmata* has both reduced growth and a loss of reproductive potential (Lirman 2000a) and regeneration in this species seems to require a limited, initial amount of energy the capacity for which might be exceeded in larger lesions, resulting in lesion retention (Lirman 2000b). *A. palmata* with neoplasms regenerates lesions more slowly than unaffected corals (Bak 1983).

Observational and experimental field studies on scleractinian regeneration have noted high variability in the time to regeneration in corals in a variety of environmental conditions (Denis et al. 2013; Fisher et al. 2007; Henry and Hart 2005; Horricks et al. 2019, 2020; Rodríguez-Villalobos et al. 2016; Sabine et al. 2015; Work and Aeby 2010). These studies have used gross measures of tissue regeneration or growth, yet liquid chromatography-mass spectrometry (LC-MS) (Casey et al. 2017) and proteomic techniques can also be used to characterise the process of tissue regeneration (Horricks et al. 2019, 2020). Tandem mass tags (TMT) (Thompson et al. 2003) have been used to quantify differentially abundant proteins in scleractinian corals during bleaching (Weston et al. 2015), the acquisition of endosymbionts (Cziesielski et al. 2018) and tissue regeneration (Horricks et al. 2019, 2020).

The present study set out to determine: 1) temporal variation in differentially abundant proteins during tissue regeneration in *R. florida*; and 2) differentially abundant proteins common to *R. florida* and the scleractinian coral *Montastraea cavernosa*.

Materials and methods

Husbandry conditions

Coral polyps (2.54 cm^2±1.01, mean±SD) on rocky substrate were imported from the wild in Florida (Reef Wholesale, Mississauga, ON) to the Hagen Aqualab (University of Guelph) and acclimated for 2 weeks. After removal from the rocky substrate, polyps were placed onto loose coral skeletal rubble in a shallow 90 L aquarium connected to a 1100 L recirculation system.

Seawater was prepared using synthetic sea salt (Instant Ocean, Spectrum Brands, Blacksburg, VA, USA) dissolved in deionised water to a salinity of 34±1 parts per thousand. Salinity was checked daily and water lost to evaporation was replenished with deionised water. Water temperature was maintained at 25.5±1°C using two EHEIM thermocontrol 300 W heaters (EHEIM, Buffalo, NY, USA). Nitrate (NO3) levels were tested weekly using a Hach DR2800 Spectrophotometer (Hach Company, Loveland, CO, USA) and ranged from 0–10 mg/L. pH levels were maintained at 8.2–8.3 (accumet AB15 Plus pH meter; ThermoFisher Scientific Inc., Ottawa ON, Canada) with biweekly addition of sodium carbonate (Reef Supplies, Montreal QC, Canada). Five percent system volume water changes were conducted weekly. A refugium of *Halimeda opuntia* and a recirculating protein skimmer (Coralvue, Slidell, LA, USA) were in circuit. Corals were maintained under Ecotech Radion XR30 Gen 3 lights (Ecotech, Allentown, PA, USA) emitting 100% ultraviolet, royal blue and blue, 15% white, 20% green and 25% red wavelengths at 30% brightness. Photosynthetically active radiation ranged from 80–105 µmol m-2 s-1 in the experimental tray at the depth of the corals.

Experimental conditions

Twenty-four *R. florida* polyps with one mouth per polyp were randomly distributed in eight separate 11.5 cm × 14.5 cm cells in the experimental tray. Cells within the experimental tray were made of 1 cm^2 polystyrene egg crate and used to keep coral polyps of interest in the same cell. Photographs were taken of each cell with a ruler immediately adjacent to the cell using a Nikon D7100 with a Sigma 8–16 mm wide angle lens. Images were imported to ImageJ (National Institutes of Health, Bethesda, MD, USA; Abràmoff et al. 2004) where the scale bar to pixel ratio for each image was calibrated and the surface area of each *R. florida* polyp was determined using the freehand line tool.

Individual *R. florida* polyps were bisected across the mouth using a sterile scalpel blade. One half of each bisected polyp was processed for proteomics (see below) and the remaining half of all polyps were returned to their assigned cells and allowed to regenerate. Regenerating *R. florida* tissue was collected at 6, 12 and 24 hr, and 2, 4, 8 and 14 d post-bisection. A separate experiment was repeated with twelve *R. florida* with samples collected from three individual polyps at 6 and 24 hr and 4 d post-bisection. Fragmented polyps for proteomics were immediately rinsed in deionised water, placed in a labelled 1.5 mL centrifuge tube and flash-frozen using liquid nitrogen. All flash-frozen *R. florida* tissue was stored at -80°C at the University of Guelph. Photographs were captured immediately before bisection, immediately after bisection (0 hr), at 12 and 24 hr and every 2 d for 14 d following bisection.

Soluble protein extraction

Protein was extracted from coral tissue following Barneah et al. (2006) and modified as described in Horricks et al. (2019, 2020). Protein concentrations were determined using a NanoDrop (ThermoFisher). Extracted protein from the three polyp halves in each cell undergoing regeneration from the first experiment were pooled. To increase confidence in the LC-MS results, extracted protein from three polyp halves in each cell undergoing regeneration in the second experiment were not pooled and were analysed as individual samples immediately following bisection.

Mass spectrometry

Extracted protein samples were submitted on dry ice to the SPARC BioCentre at the Toronto Hospital for Sick Children for TMT labelling and mass spectrometry. The proteins were reduced and alkylated and precipitated with acetone as described in Horricks et al. (2019, 2020). Fifty µg of pooled protein from each time point was labelled using 21 µL of TMT 11-plex. Samples were analysed on an Orbitrap analyser (Q Exactive, ThermoFisher) outfitted with a nanospray source and EASY-nLC 1200 nano-LC system (ThermoFisher).

Data analysis

Data files were analysed using PEAKS X+ (Bioinformatics Solutions Inc., Waterloo, ON) as described in Horricks et al. (2019, 2020) to quantify differentially abundant proteins using the MaxQuant algorithm (Cox and Mann 2008). De novo assemblies were made in PEAKS (Zhang et al. 2012) and National Center for Biotechnology Information (NCBI) database entries for Cnidaria were searched (https://www.ncbi.nlm.nih.gov/protein/?term=txid6073[Organism:exp]). Data from the two TMT-labelled mass-spectrometry runs (pooled versus individual polyps) were combined and normalised using control samples common to each run. Fold change (FC) values relative to the initial protein abundance following bisection (0 hr) were calculated to describe the differential abundance of proteins during tissue regeneration. Mean FC values and their standard deviation were calculated.

Back-translated sequences of all differentially abundant proteins were submitted to the NCBI basic local alignment search tool (BLAST; Altschul et al. 1990) service using an expectation value (e-value) of 1e-03 to confirm that they had originated from coral tissue. Gene ontology terms were mapped to correctly identified

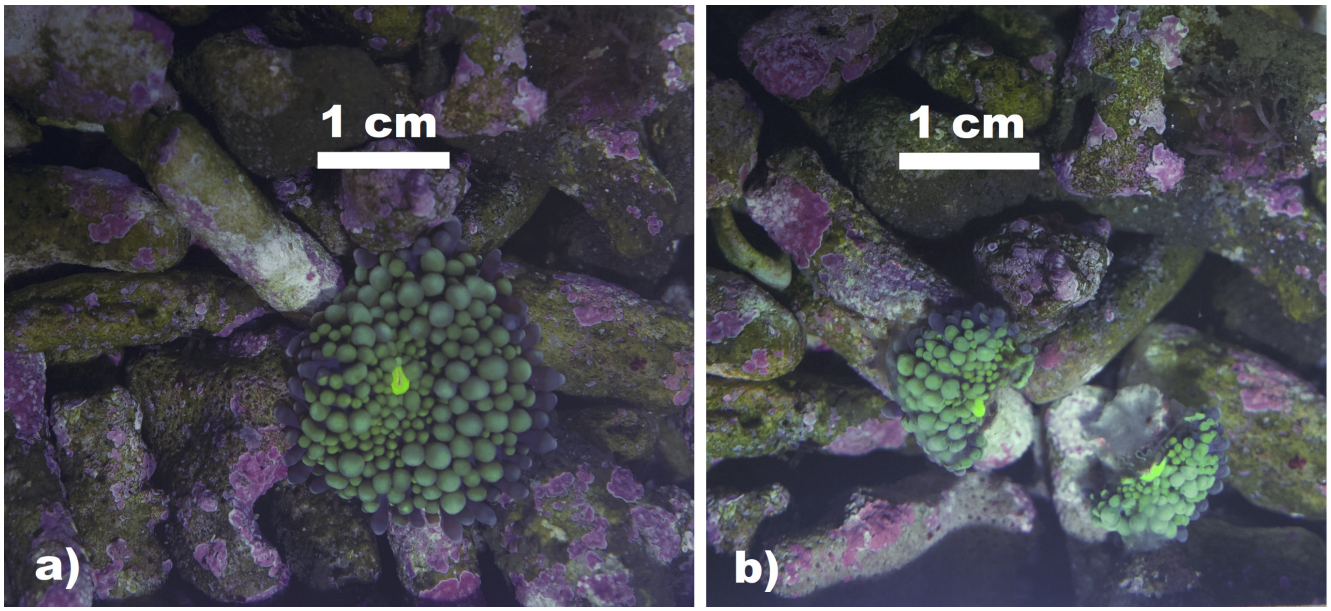

Figure 1. *Ricordea florida* polyps (a) pre- and (b) immediately post-bisection at the Hagen Aqualab, University of Guelph, Guelph, ON

sequence IDs in Blast2GO using confidence score estimates (Conesa et al. 2005). The Blast2GO mapping and annotation feature was used to annotate all sequences with further gene ontology terms by searching an additional nine protein databases and merging these results and those returned by the native Blast2GO search. To be included as a major gene ontology category, a category was required to contain at least 10% of the total number of sequences in the dataset.

Results

R. florida polyps were 0.79 cm^2±0.40 (mean±SD) immediately following bisection. Gross photography showed that the polyps reduced in size and contracted by more than 5% of their original surface area following bisection (Figure 1). All polyps regenerated in a similar manner. Coral polyps began to re-circularise as early as 6 hr following bisection. The edges of the lesion folded inwards around the mouth until the polyps had completely re-circularised. Polyps contouring to the rugose coral rubble prevented accurate measurements of surface area for the remainder of the experiment, however, images could still be used to determine the time to re-circularisation. Coral polyps required 2 to 8 d to re-circularise (Table 1). Coral polyps were defined as 'closed' once they had completely re-circularised and the lesion resulting from the bisection was no longer visible. The majority of coral polyps had completely regenerated within 4 d following bisection while all coral polyps had completely re-circularised within two weeks post-bisection (Table 1).

A total of 2608 peptide sequences were reliably identified using the selection criteria, which corresponded to 1192 unique proteins common between the two LC-MS runs. Of these, there were 100 proteins that were significantly differentially abundant over the course of the 14-day experimental period (Figure 2). Since the majority of coral polyps had re-circularised within 4 d

Table 1. Re-circularisation of *Ricordea florida* polyps during the experimental period

Time point	Number of polyps	Number re-circularised	Re-circularised (%)
0 hr	24	0	0.0
6 hr	21	0	0.0
12 hr	18	0	0.0
24 hr	15	4	26.7
2 d	12	5	41.7
4 d	9	5	55.6
6 d	9	5	55.6
8 d	6	4	66.7
10 d	6	4	66.7
12 d	6	5	83.3
14 d	3	3	100.0

following bisection (Table 1), only differentially abundant proteins at the 6 hr, 24 hr and 4 d time points were examined. Twenty-two differentially abundant proteins common to both regenerating pooled and individual polyps were examined for their variability in three individual polyps at these time points (Figure 3). Apart from green fluorescent proteins, there was very limited individual variation in differentially abundant proteins during regeneration.

Following gene ontology annotation, two proteins could not

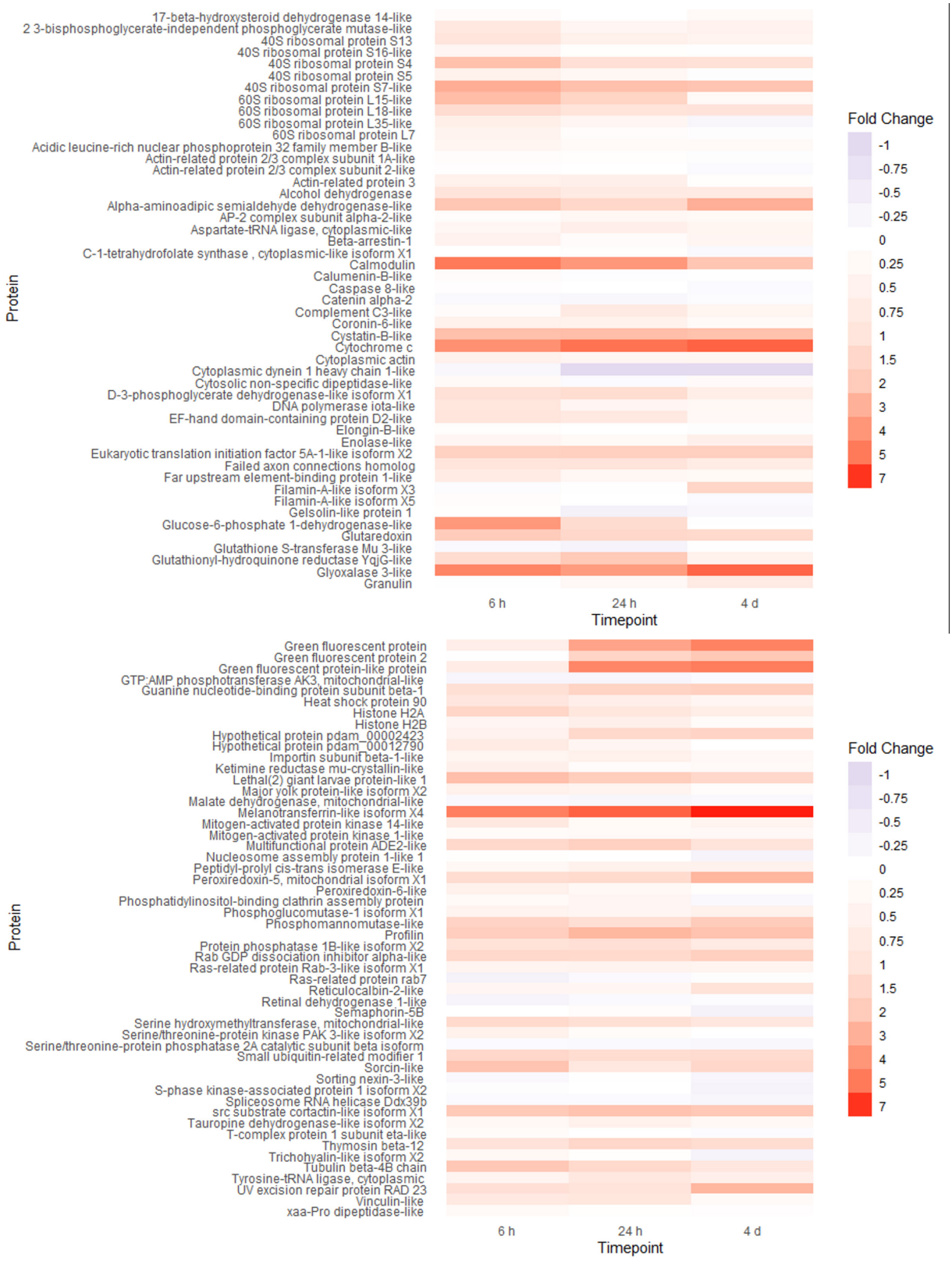

Figure 2. Heatmap showing fold change of differentially abundant *Ricordea florida* proteins common to both experiments at sampling time points 6 hr, 24 hr and 4 d relative to hr 0 (time of bisection)

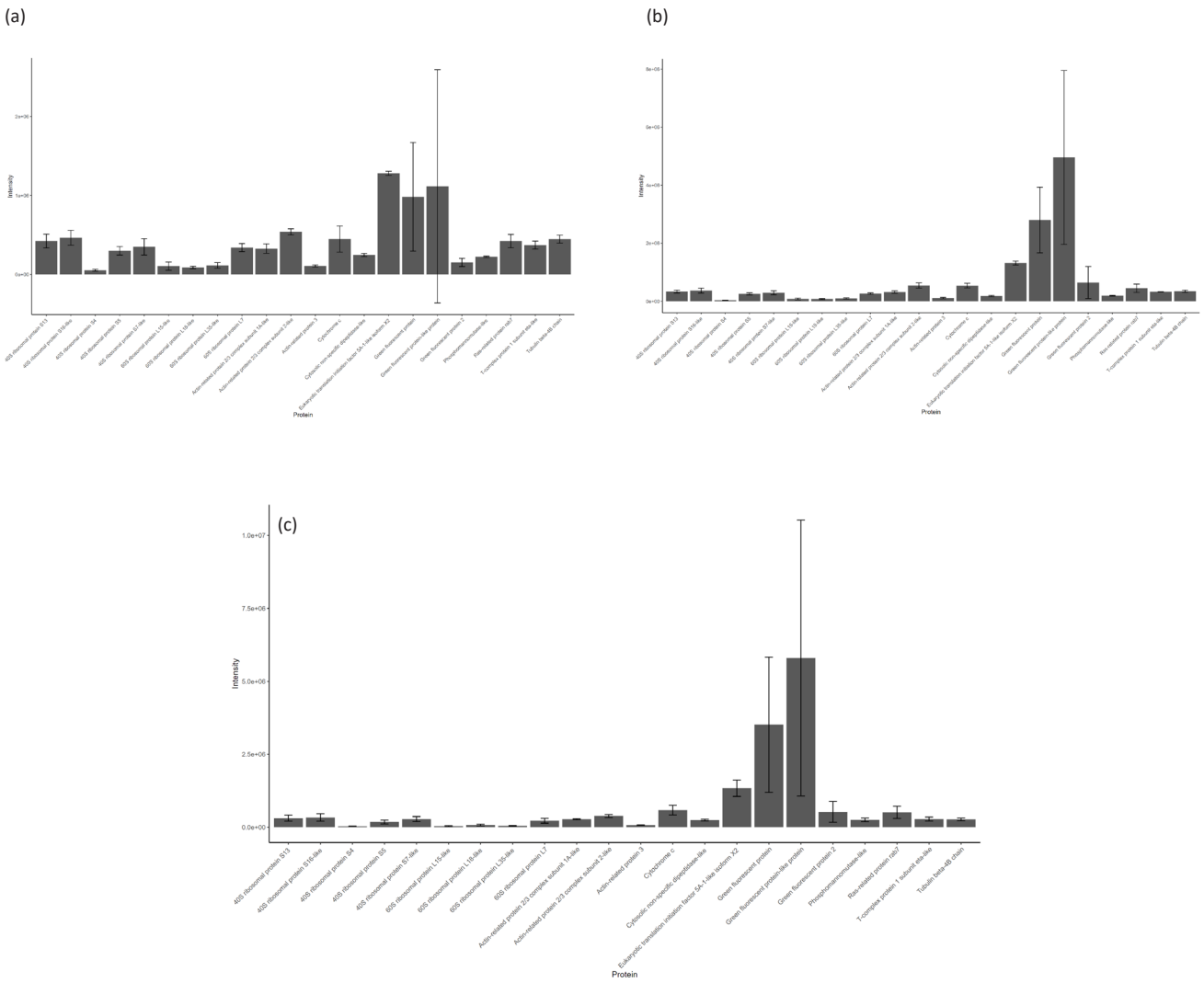

Figure 3. Mean intensity of expression (±SD) for selected differentially abundant *Ricordea florida* proteins at sampling time points (a) 6 hr, (b) 24 hr and (c) 4 d (n=3 individuals per time point)

be matched with any gene ontology terms. Thirty-five percent of protein sequences were matched to ≥10 gene ontology terms while one protein (calmodulin) matched to 43 gene ontology terms. The biological process gene ontology categories containing the highest proportion of sequence scores were translation (24%), oxidation-reduction (17%), transport (13%) and regulation of cellular processes (12%). The mean FC of differentially abundant proteins was 0.858±1.23.

Thirty-three proteins that were significantly differentially abundant in regenerating *R. florida* tissues were the same as those previously identified to be significantly differentially abundant in regenerating *M. cavernosa* tissues (Table 2; Horricks et al. 2019, 2020). Of the proteins that were significantly differentially abundant and common between *R. florida* and *M. cavernosa*, the majority (24%) were related to oxidative stress and cytoskeletal processes (18%).

Discussion

Like scleractinians, corallimorpharians contain symbiotic zooxanthellae, however, known symbiont proteins were not detected in the present study as the extraction methodology used was purposefully very gentle. Ongoing experiments use more aggressive extraction, expanding the proteome detected to be more likely to include symbiont proteins (unpublished results). Since corallimorpharians are related to scleractinians, some common proteins, particularly those involved in cytoskeleton and oxidative stress during regeneration, were expected. The detection of 33 differentially abundant proteins in common between *R. florida* and *M. cavernosa* was rewarding, particularly as some markedly differentially abundant proteins are responsible for critical cellular processes and may prove useful as regeneration biomarkers.

Table 2. Proteins that were differentially abundant during tissue regeneration in both *Ricordea florida* and *Montastraea cavernosa.* e-values smaller than 2.225074e-308 are represented as 0.0. *A.: Acropora, *A.: Actinia, D.: Dendronephthya, E.: Exaiptasia, N.: Nematostella, O.: Orbicella, P.: Pocillopora, R.: Ricordea, S.: Stylophora, T.: Trichinella*

Accession ID	Protein	Role	Species	e-Value	% Identity
1270048700	Cytochrome c	Apoptosis	*S. pistillata*	4.00E-74	100
1778058688	Rab GDP dissociation inhibitor alpha-like	Apoptosis	**A. tenebrosa*	0	100
1176107158	Failed axon connections homologue	Axon	*O. faveolata*	0	100
1270055404	UV excision repair protein RAD 23	Catabolism	*P. damicornis*	0	100
1500188362	xaa-Pro dipeptidase-like	Catabolism	*S. pistillata*	0	100
1005425547	Histone H2A	Cell proliferation	*S. pistillata*	0	100
1263125240	Histone H2B	Cell proliferation	*A. digitifera*	1.00E-154	100
999969978	Guanine nucleotide-binding protein subunit beta-1	Cellular signalling	*E. pallida*	0	100
156363871	Actin-related protein 2/3 complex subunit 1A-like	Cytoskeleton	*P. damicornis*	0	96.29
1176070375	Actin-related protein 3	Cytoskeleton	*O. faveolata*	0	100
1176078697	Gelsolin-like protein 1	Cytoskeleton	*O. faveolata*	2.00E-150	100
1263121419	Lethal(2) giant larvae protein-like 1	Cytoskeleton	*S. pistillata*	0	100
1263122016	Profilin	Cytoskeleton	*N. vectensis*	8.00E-99	100
1500189763	Tubulin beta-4B chain	Cytoskeleton	*S. pistillata*	0	100
1176077959	Enolase-like	Detoxification	*S. pistillata*	0	100
1270061878	Glyoxalase 3-like	Detoxification	*O. faveolata*	0	100
1176098222	Vinculin-like	Extracellular matrix	*O. faveolata*	0	100
122063216	Calmodulin	Inflammation	*T. pseudospiralis*	2.00E-100	97.99
1778032378	Peptidyl-prolyl cis-trans isomerase E-like	Inflammation	**A. tenebrosa*	0	100
1005435265	Melanotransferrin-like isoform X4	Membrane traffic	*A, digitifera*	0	100
1176120696	Ras-related protein Rab-3-like isoform X1	Membrane traffic	*O. faveolata*	0	100
1603789294	Ras-related protein Rab7	Membrane traffic	*D. gigantea*	1.00E-150	100
1176093509	Calumenin-B-like	Multiple	*O. faveolata*	0	100
19982622	17-beta-hydroxysteroid dehydrogenase 14-like	Oxidative stress	**A. tenebrosa*	0	100
19982635	Green fluorescent protein	Oxidative stress	*Discosoma* spp.	3.00E-139	82.25
21303780	Green fluorescent protein 2	Oxidative stress	*R. florida*	7.00E-44	83.98
1227044464	Green fluorescent protein-like protein	Oxidative stress	*R. florida*	8.00E-171	99.57
1270065268	Glutathione S-transferase Mu 3-like	Oxidative stress	**A. tenebrosa*	5.00E-159	100
1778061585	Heat shock protein 90	Oxidative stress	*A. tenuis*	0	100
1778070517	Peroxiredoxin-5, mitochondrial isoform X1	Oxidative stress	*N. vectensis*	1.00E-151	100
1806716423	Peroxiredoxin-6-like	Oxidative stress	*S. pistillata*	5.00E-158	100
1176071648	Cytosolic non-specific dipeptidase-like	Protein catabolism	*O. faveolata*	0	100
1176118675	Eukaryotic translation initiation factor 5A-1-like isoform	Protein synthesis	*O. faveolata*	3.00E-110	100

The repeated pattern of high FC values at 6 hr, 24 hr and 4 d post-transection in most, but not all regenerating corals, suggests that there are events critical to tissue regeneration occurring at these time points. The limited variability in differentially abundant proteins detected between individuals undergoing regeneration supports their suitability as potential biomarkers. Apart from the 6 hr collection, all samples were obtained at the same time of day, likely limiting variability in proteins that would occur with diurnal or temporal variation. The 4 d time point specifically, when greater than 55% of remaining polyps had re-circularised, appears to be critical for tissue regeneration in *R. florida*.

Oxidative stress

Heat shock protein 90 is a marker for stress in many organisms (Chen et al. 2006), including corals (Horricks et al. 2019, 2020; Rossi and Snyder 2001). It was significantly differentially abundant during the first 24 hr of tissue regeneration (FC 1.94) and remained elevated throughout the experiment (FC>1.3). Cell death releases

oxidative compounds and free radicals must be neutralised to allow regeneration. Fourteen proteins associated with oxidative stress were significantly differentially abundant over the course of the experiment, including eight previously identified in regenerating *M. cavernosa*. Of particular note, green fluorescent proteins are known to act as antioxidants (Palmer et al. 2009) and control free radicals in cnidarians (D'Angelo et al. 2012). Three different green fluorescent proteins were significantly differentially abundant in regenerating *R. florida* tissue and all showed a similar pattern of increasing abundance that peaked at 4 d (average FC 4.9).

The protein cytochrome c oxidase had the second highest FC in the present study (FC>5.1) and peaked 4 d after bisection (FC 6.77). Cytochrome c oxidase has been identified as a marker for apoptosis in mammals (Liu et al. 1996), programmed cell death (Gao and Dou 2001) and is critical for energy metabolism in corals (Rosic et al. 2014). Once a regenerating corallimorpharian polyp re-circularises (~4 d), histological sections show cell death where the bisected tissue fronts merge and copious cell differentiation and tissue remodelling (Vroom 2016).

Cytoskeleton

The diurnal regulation of coral tissue requires the involvement of actins and tubulins (Mayfield et al. 2010). These proteins are highly likely to be involved in the reorganisation of coral tissue during regeneration and were commonly differentially abundant. Actin-related protein 3 was positively differentially abundant throughout tissue regeneration in *R. florida* and was also significantly differentially abundant in regenerating *M. cavernosa* (Horricks et al. 2019). The cytoskeletal protein cortactin acts to reorganise cytoskeletal actin (Uruno et al. 2001). A cortactin-like protein had increased differential abundance throughout the regeneration process (FC>2.0) with the largest increase 24 hr following bisection (FC 3.32). The T-complex protein 1 followed similar patterns of differential abundance, with the largest increase also at 24 hr (FC 2.32). T-complex proteins are molecular chaperones required for the proper folding of actin and tubulin (Yaffe et al. 1992). Tubulin beta-4B, required for microtubule formation in mitosis, was significantly differentially abundant at 24 hr post-bisection (FC 2.11). The highest FC in proteins related to cytoskeletal processes was seen in profilin (FC 2.74) at 24 hr following bisection. This protein has been observed in *M. cavernosa* (Horricks et al. 2019, 2020).

The consistent pattern of cytoskeletal proteins increasing in differential abundance at 24 hr following bisection suggests that the rapid increase in cytoskeletal mobilisation is critical for the relatively fast healing of corallimorpharian lesions compared to scleractinian lesions (Horricks et al. 2019, 2020). Additionally, proteins related to cytoskeletal processes comprised the second largest proportion (18%) of all differentially abundant proteins in the present study.

Membrane traffic

Seven unique proteins that are involved in the regulation of membrane traffic were significantly differentially abundant in regenerating *R. florida* tissue, including the ras-related protein Rab7. Ras-related protein Rab7 decreased rapidly 24 hr following bisection (FC -0.35). This protein is fundamental to lysosomal biogenesis and has been implicated in the apoptotic pathway in mammals (Guerra and Bucci 2016). The decreased abundance observed in the present study 24 hr following bisection allows for a similar role in cnidarians.

Metabolism

Apart from cytochrome c oxidase, four metabolism-related proteins were significantly differentially abundant including a phosphomannomutase-like protein. Phosphomannomutase is fundamentally important across taxa and is known to catalyse the interconversion of mannose-6-phosphate and mannose-1-phosphate (Qian et al. 2007). Mannose-1-phosphate is essential in the synthesis of GDP-mannose which is in turn essential for protein glycosylation, sorting and secretion, and the maintenance of a functional endomembrane system (Qian et al. 2007). The high abundance observed at 4 d is consistent with the demands of cell differentiation and tissue remodelling.

Protein synthesis

Eukaryotic translation factors are critical for protein synthesis (Henderson and Hershey 2011). Eukaryotic translation initiation factor 5A increased in abundance 6 hr following coral polyp bisection (FC 2.73) and maintained a high FC (FC>2.8) throughout the experiment. The high FC attained in such a short amount of time following lesion creation demonstrates the rapid response by *R. florida* to bisection. Following elongation, tRNA must bind to a ribosome before protein synthesis can begin. Nine different ribosomal proteins were significantly differentially abundant during tissue regeneration and three of these (40s ribosomal protein S7, 60s ribosomal protein L15 and 40s ribosomal protein S4) had the highest abundance 6 hr following lesion creation (FC 3.11, 2.55 and 2.39 respectively).

Guided by the two-week tissue regeneration timeframe identified by Vroom (2016) under laboratory conditions, the present study applied the use of proven proteomic techniques (Horricks et al. 2019, 2020) developed from Caribbean scleractinian corals regenerating in the wild to study tissue regeneration using a Caribbean corallimorpharian *Ricordea florida* in a controlled laboratory setting. Cytoskeletal mobilisation and tissue reorganisation in the first few days following lesion creation appear to be critical for regeneration success. The identified differentially abundant proteins common to regeneration in a corallimorpharian and a scleractinian provide a basis for continued mechanistic study. Identification of protein biomarkers common to regenerating corals will allow the development of tools, e.g. antibodies, to track protein expression during tissue remodelling and stressful events. This will also allow identification of innate immune molecules responsible for defence since the corals are exposed to the environment following bisection. Ongoing experiments include an examination of the success of different techniques of fragmentation of corallimorpharians and scleractinians in captivity and the associated proteomes. Multiple experiments with different species are required to identify valid biomarkers for future use.

A more detailed understanding of fragmentation and regeneration in corallimorpharians is needed. While sexual reproduction occurs in the wild, clonal division forming large colonies is the dominant method of population expansion (Chen et al. 1995). In captivity, sexual reproduction has not been recorded, and population increases rely exclusively on asexual reproduction, or fragmentation, for propagation. Therefore, improved understanding of regeneration can contribute to improved corallimorpharian propagation in captivity, which may reduce demand from the aquarium industry for wild harvesting.

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