Seasonal Temperatures Reveal Unique Markers in the Endangered Coral species *Acropora Palmata*

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Research Article

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Abstract

In recent decades, coral reefs have been deteriorating due to global warming compounded with many other anthropogenic-induced stressors worldwide. One of the most revealing signs of climate change is the increase in seawater temperature, which is affecting the metabolic functions of corals and many other marine organisms. To assess potential metabolic responses induced by high temperatures, the proteomic profile of the most iconic Caribbean coral species *Acropora palmata* was investigated during two seasons 2014 and 2015, in Puerto Rico. A combination of two-dimensional gel electrophoresis (2D-GE) and mass spectrometry was used to analyze protein expression patterns during the cool dry season as compared to hot wet season in *A. palmata*. A total of 527 proteins having a significant differential expression response to seasonal temperature were retrieved. Results indicated that changes in temperature of 3 to 3.5°C, were enough to alter *A. palmata's* proteome, inducing significant changes in key metabolic, enzymatic, translation, and apoptosis processes among other important cellular functions. These analyses provide insights to discover heat stress candidate biomarkers of *A. palmata* resistance against low and high temperatures during the two different seasons. Subsequently, the transcription level of five genes in response to high/low temperatures during the two seasons was determined by RT-PCR. In summary, we have identified key proteins expressed during natural temperature changes, indicating the ability of *A. palmata*'s metabolism to acclimatize to fluctuations of seasonal temperature and the potential to respond to climate change driven thermal anomalies.

Introduction

The Caribbean has more than 60 species of scleractinian corals, some of which began to decline greatly in abundance in the early 1980s producing significant ecological changes in coral reefs (Precht et al. 2004; Bruno et al. 2007; Leggat et al. 2007; Weil et al. 2009; Jackson et al. 2014; Alvarez-Filip et al. 2015; García-Urueña and Garzón-Machado 2020). Coral reefs are one of the most diverse and valuable marine ecosystems on earth and have continued declining over the past three decades, more specifically during and after the thermal anomalies (heat stress) events of 1998, 2005-06, 2010-11, 2015 and 2017 (Aronson et al. 2002; Bruno et al. 2007; Middlebrook et al. 2010; Eakin et al. 2010; Weil and Rogers 2011; Burge et al. 2014; Pratchett et al. 2015; Lough et al. 2018; Muñiz-Castillo et al. 2019). A global warming of 1.5°C above pre-industrial levels could result in the loss of 70 to 90 % of coral reefs with even further losses (up to 99%) with 2°C temperature increases (Intergovernmental Panel on Climate Change -IPCC 2018). Under these IPCC global warming scenarios, many threatened and endangered coral species could disappear in the next 20–50 years (IUCN 2014; Logan et al. 2014; Hoegh-Guldberg et al. 2017; Olguín-López et al. 2018).

There is little doubt that reef-building corals will experience higher sea temperatures in the span of the next few decades under the IPCC scenarios, however, they also experience large fluctuations of sea temperatures and other environmental variables during diel and seasonal cycles. Seasonal variation in Puerto Rico (Rios-Jara 1998) as manifested in sea temperatures is an important environmental variable for reef building corals; the highest temperatures been recorded during the hot wet season (September

and October, > 30 ºC) are conducing to thermal stress (Zolg 2006; Portune et al. 2010; DeSalvo et al. 2010; Souter et al. 2011; McGinley et al. 2012; Barshis et al. 2013; Alemu and Clement 2014; Carballo et al. 2020), leading to physiological disruption, bleaching and diseases (Jones et al. 1998; Winter et al. 1998; Banin et al. 2000; Morelock et al. 2001; Gardner 2003; Aronson and Precht 2006; Hoegh-Guldberg et al. 2007, Harvell et al. 2009; Toth et al. 2019; Caballero-Aragón et al. 2020; Cramer et al. 2020).

The Caribbean Elkhorn coral *Acropora palmata* (Lamarck, 1816) is one of the most ecologically and geologically dominant reef-building species (Bruno et al. 2007; Pratchett et al. 2015; Van Woesik and Randall 2017; Hagedorn et al. 2018) that has persisted since late Pliocene (McNeill et al. 1997; Irwin et al. 2017; Mudge et al. 2019). This implies, that they have survived and thrived through many substantial environmental changes and possibly providing some hope that they may survive further anticipated climate change (Torda et al. 2017; Hughes et al. 2018; García-Urueña and Garzón-Machado 2020). Due to the high abundance of *A. palmata* in shallow waters (0–5 m), they are constantly exposed to long periods of high temperatures and ultraviolet radiation during the year and especially during the warmer months of the year. *Acropora palmata* as well as other shallow water reef-building corals are often physiologically stressed leading to bleaching, diseases, or death (Patterson Sutherland and Ritchie; Bruckner and Bruckner 2006; Weil and Croquer 2009; Miller et al. 2009; Eakin et al. 2010; Jackson et al. 2014; Rosic et al. 2014; Montilla et al. 2016; Ricaurte et al. 2016; Cramer et al. 2020).

Proteomics can provide an overall view of protein functions affected by climate change (Tomanek 2014) and the application of proteomic techniques to marine sciences has increased in recent years (e.g., Serafini et al. 2011; Weston et al. 2012; Drake et al. 2013; Ricaurte et al. 2016). To evaluate the effect of seasonal temperature fluctuations on protein expression in *A. palmata*, the proteomic profile of *A. palmata* colonies was examined during two seasons in a tropical setting. are immersed in water, a much better conductor of heat than air, therefore, This implies that fufurther research would needs to be done toconducted to determine the chronic effects of continuously fluctuating temperatures on aquatic organisms. specifically,Natural seasonal changes in temperature changes effects that remain to be studied.provide an appropriate experimental setting to test the effects of temperature changes in marine organisms, *in situ.* We assessed protein expression patterns during the cool dry (January and February) and the hot wet season (September and October) in the Natural Reserve of La Parguera, southwest coast of Puerto Rico. Here, we predicted that colonies collected during the hot wet seasons of 2014 and 2015 will exhibit altered differential protein abundances as compared to the those during the cool dry seasons. This study provides new insights into the protein profile of *A. palmata* and a metabolic pathway for acclimation in response to seasonal temperatures as well as environmental changes (Tomanek 2010; Reyer et al. 2017). The proteomic flexibility of *A. palmata* during seasonal environmental variations give us a glimpse on how the species may respond to larger fluctuations expected in the future (Schoepf et al. 2015; Hoegh-Guldberg et al. 2017; Scheufen et al. 2017; IPCC 2018).

Materials And Methods

All the methodology steps including proteomics workflow were summarized in Figure 3.

Study area and sampling locations

The study was carried out at the inner-shelf reef Enrique (17.57.15 N, 67.02.55 W) and at the mid-shelf reef San Cristobal (17.56.30 N, 67.04.45 W) (Fig. 1) during the months of January -February, and September-October of 2014-2015 representing the cool dry and hot wet seasons, respectively. The collection of *Acropora palmata* colonies was made near the laboratories of Magueyes Island in the Natural Reserve of La Parguera, on the southwest coast of Puerto Rico.

Six colonies of *A. palmata* were sampled from each reef and tagged with a specific number. (See Supplementary Material 1 and 4). The same colonies were re-sampled during 2014 and 2015. All coral colonies were collected under permission No. O-VS-PVS15-SJ-00668-22042014 issued by the Department of Natural and Environmental Resources of Puerto Rico. The temperatures were measured in situ with a Hobo-pro every hour (Fig. 2).

Colonies of *A. palmata* were sampled at a depth of 1 to 2 m separated by distances of at least 3 m to reduce the possibility of sampling genetic clones. A fragment (3 x 3 cm) was removed from each colony with the help of a chisel and a hammer and placed in plastic bags where they were kept on ice and transported to the laboratory. Each fragment was then broken into three small pieces of an approximate size of 2 cm^2 (n = 3 biological replicates per colony per sampling season; in total 72 biological samples) (See Supplementary Material 1), and proteins were extracted as previously described (Ricaurte et al. 2016). Briefly, each fragment was placed in a mortar to be pulverized in 1 ml of rehydration buffer (9.5 M urea, 2% CHAPS, 1% DTT). The homogenized coral tissue was placed in a 1.5 ml tube and lysed under ice sonication, followed by slow stirring at room temperature for 1 hour. Samples were centrifuged at 12,000 rpm for 30 minutes at 4°C, and the supernatant with the upper layer without zooxanthellae was placed in a new 1.5 ml Eppendorf tube. Protein concentration was estimated using the Bradford method (Bradford 1976), following the manufacturer's recommendations (Invitrogen, Carlsbad, CA, USA).

High-resolution two-dimensional gel electrophoresis (2D-GE)

Three replicate samples (i.e., fragments) per coral colony per season and sampling year were electrophoresed for protein separation. Two hundred micrograms of protein from A. palmata were mixed in rehydration buffer (Bio-Rad) overnight on immobilized pH gradient (IPG) strips (pH 3–10), which were then subjected to first-dimension Isoelectric Focusing (IEF) in a Bio-Rad protein IEF cell (50 µA/strip) for a total of 20,000 Vh, as previously described in Boukli et al. 2011, 2012 and Ricaurte et al. 2016.

After completion of the run, strips were reduced and alkylated for 2 × 15 min each in equilibration buffer I (6 mol L^{-1} urea, 2% SDS, 0.05 mol L^{-1} Tris–HCl, pH 8.8, 50% glycerol, and 2% [w/v] dithiothreitol [DTT]) followed by equilibration buffer II (6 mol L^{-1} urea, 2% SDS 0.05 mol L^{-1}, Tris–HCl, pH 8.8, 50% glycerol and 2.5% [w/v] iodoacetamide). The second-dimension separation was performed with a 4-20% gradient

SDS-polyacrylamide gel, to increase the range of molecular weights and sharpening bands (15 mA/gel for 15 min, then 30 mA/gel). The SDS-polyacrylamide gels were then stained with BioSafe Coomassie blue G-250 dye (Bio-Rad) for visualization and image analysis (Fig. 4).

Gel image and statistical analysis

2D gel images belonging to cool dry and hot wet seasons from A. palmata were scanned using Gel Image system (BioRad, Hercules, CA) and analyzed using PD Quest software, version 8.0.1 (BioRad) to detect significant, and consistency of the expressed proteins (See Supplementary Material 1 and 4), the gel area was defined using selected proteins bordering each side of the gels as landmark. In all cases this area corresponded to at least 95% of the total gel area. Spots across the gel replicates were matched by landmarks that label the spots present and positioned consistently, in all replicated gels. The Analysis Set, derived from three replicated gels of matched spots that were present on all the gels, was created, and the spots were analyzed and characterized. Three independent replicates were performed per sample and image analysis was carried out considering all gels. Initially, three replicated gels were analyzed by PD Quest (version 8.0.1) to normalize the gel images and created a master gel profile *Acropora palmata* samples during dry season and wet season. The master profiles representing each of these conditions were compared to determine the differences in relative protein abundance among the samples. A one-way analysis of variance (ANOVA) was conducted to compare the mean protein spot densities and test if there was any difference in the protein spot abundance among the cool dry and hot wet season groups. The differentially expressed spots (with P-values <0.05, the Shapiro–Wilk ($p < 0.0001$) and Levene's tests showing significant differences in the protein abundance time points were chosen to determine whether all proteins expressed in A. palmata were normally distributed and exhibited homogeneous variance, respectively (See Supplementary Material 2). Protein spots that demonstrated a ratio of at least 2-fold between one another were defined as differentially expressed proteins. Within this context, fold change refers to the ratio of relative abundance of a protein between the two samples. The proteins expressed during the cool dry season were compared with the proteins expressed during the hot wet season. 32 protein spots that showed significant differences in the spot densities and the spots showing at least ≥2-fold difference between the two seasons from *A. palmata* were estimated by PD Quest software. Subsequently, the 32 spots were manually excised from the gels and subjected to MALDI/TOF and database search for identification. The analysis procedure to detect the relative quantification of the real time PCR used the ΔΔCt model (Supplementary Material 3).

Proteins In-gel digestion and data analysis

Thirty-two 2D-GE spots from *A. palmata* were manually excised from Coomassie blue stained gels using a sterile scalpel and kept in sterile microcentrifuge tubes for distaining with 40% methanol/10% acetic acid, reduced with 10 mmol L^{-1} DTT in 50 mmol L^{-1} ammonium bicarbonate (AB), then alkylated with 55 mmol L^{-1} iodoacetamide in 50 mmol L^{-1} AB. The resulting gel fragments were rinsed with 50 mmol L^{-1} AB and 50% acetonitrile (ACN) and dried under a stream of nitrogen. Samples were digested with 5%

trypsin proteomics grade (w/w) (Sigma-Aldrich) overnight at 37°C and combined with an equal volume of saturated cyano- hydroxycinnamic acid in 50% ACN/0.1% trifluoracetic acid. Half of the mixture from each spot was applied to a MALDI target plate.

Mass spectrometry analysis

Mass spectrometry (MS) was performed in a MALDI-TOF/TOF 4800 mass spectrometer (Applied Biosystems) at the Durham Research Center, University of Nebraska Medical Center (Omaha, Nebraska). One microliter of in-gel digested spots from A. palmata was spotted directly onto a MALDI plate (Applied Biosystems, Foster City, CA) and co-crystallized with α-cyano-4-hydroxycinnamic acid (CHCA) matrix (5 mg/mL in 50% ACN-0.1% TFA). Droplets were dried at room temperature. Spectra acquisition and processing was performed with the 4000 series Explorer software (Applied Biosystems) version 3.5.1 in positive reflectron mode at fixed laser fluency with low mass gate and delayed extraction. External calibration was performed with a mixture of five external standards (Applied Biosystems). Peptide masses were acquired by steps of 50 spectra for masses between 800 to 3000 Da. MS spectra were summed from 1000 laser shots by an Nd-YAG laser operating at 355 nm and 200 Hz. MS/MS spectra were acquired in 1-kV positive mode, and 1000 shots were summed in increments of 50. Database searching was realized using the MatrixScience website (www.matrixscience.com) for MS and MS/MS interrogations on coral proteins from Swiss-Prot databank (www.expasy.org). The search parameters were as follows: carbamidomethylation for cysteines and oxidation for methionines as variable modifications, one missed tryptic cleavage was permitted, and mass accuracy tolerance was set at 100 ppm for precursors and 0.5 Da for fragments.

RNA extraction

Total RNA was extracted from living tissue obtained from three representative colonies of A. palmataby direct application of 1 ml of TRIzol® (Invitrogen, Carlsbad, CA, USA) to ~10 cm^2 of coral surface tissue as a modification of the manufacturer's protocol (Chomzynksi 1987). All coral fragments of *A. palmata* from cool dry and hot wet seasons were placed in beakers and washed in volumes of TRIzol® for one hour on a gyratory shaker. Washing in TRIzol® was the equivalent to the homogenization step as per manufacturer's instructions. After the TRIzol® wash, the tissues were divided into 1 ml working volume aliquots and the RNA extraction protocol was completed for an individual replicate/sample. RNA concentrations were estimated by excitable fluorescence at 485 nm (RiboGreen, Molecular Probes, Portland, OR, USA). Integrity of total RNA was confirmed by electrophoresis of an aliquot of each sample on a 1% formaldehyde agarose gel (Sambrook 1987). Total RNA was further purified by DNase I digestion followed by phenol/$CHCl_3$ extraction (Message Clean®, GenHunter, Nashville, TN, USA).

Primer design and RT-PCR Validation

Five primer pairs were designed with Primer3 software (v.0.4.0), for the reverse transcription of five target genes based on their expression and cellular function in A. palmata such as cytochrome P450, pax C, minicollagen, acropsin and caspase 8 (Table 1, Fig. 6). The RNA (2 µg) from A. palmata was reverse-transcribed using SsoAdvanced™ Universal SYBR® Green Supermix (Biorad) with the corresponding primers (Table 1). Semi-quantitative PCRs were performed in 20 μL^{-1} reaction volumes using PCR Master Mix (Biorad) in a Master Cycler (Eppendorf) using the following cycling parameters: 98°C for 30 s; and 35 cycles of 98 °C for 15 s and 60°C for 30 s, followed by 60°C for 30 s. Amplicon products were separated on 2% agarose gels. After confirming that each primer-pair only amplified a single PCR product of the expected size quantitative real-time PCR (qPCR) was performed using a CFX96 Touch™ Real Time System (Biorad). Reactions were performed in triplicates using 20 μL^{-1} reaction volume with 2 μL^{-1} of cDNA SYBR Green PCR Master Mix (Qiagen) and 30 $pmoL^{-1}$ of each primer. PCR conditions were 50°C/2 min and 95°C/10 min, followed by 40 cycles of 95°C/15 s and 60°C/1 min.

Results

Field and experimental data

During 2014 and 2015, in the inner-shelf reef Enrique (Fig. 1, Fig. 2A), the sea surface temperatures (SSTs) fluctuated from a low of 26.7°C to a high of 29.8°C during the cool dry and hot wet seasons, respectively. In the mid-shelf reef San Cristobal, the SSTs were similar, fluctuating between 26.6°C to 30.0°C in both seasons, respectively (Fig. 1, Fig. 2B).

Proteomics analysis of Acropora palmata **colonies collected during the cool dry and hot wet seasons**

The sampled *A. palmata* colonies from Enrique reef revealed 545 differentially expressed proteins in the two seasons; 291 were upregulated, 120 downregulated and 67 were common proteins. With regards to San Cristobal reef, the number of differentiated proteins increased to 1070 between the two seasons, where 468 were upregulated, 360 downregulated, and 121 common proteins (Fig. 5).

A conservative two-fold selection threshold was applied by PD Quest software to control the number of 2D gel spots. Thus, only the more prominent protein changes corresponding to 32 differentially expressed proteins were selected and subsequently excised from the 2D gels, tryptic digested and processed for characterization and protein identification by MALDI-TOF/TOF mass spectrometric analysis. Proteins expressed in *A. palmata* were classified into 10 categories according to their main biological function associated with temperature stress (Fig. 5) and mechanistically proposed signaling pathway compatible with protein expression changes measured in this study (Fig. 7). Moreover, protein-protein interaction networks functional enrichment analysis through STRING database (https://string-db.org/) allowed to categorize proteins with high confidence scores based on the Gene Ontology (GO) as highlighted in Supplementary Material 5. Among the upregulated proteins, there were functions associated with stress response, (i.e., heat shock transcription factor and glutathione S-transferase), cell signaling (i.e., minicollagen and alpha i G protein) and cell cycle (i.e., calmodulin). Downregulated proteins included

proteins related to cytoskeletal binding (i.e., gelsolin and beta-tubulin), UV (green fluorescent protein and ATP synthase F0 subunit 6), immunity-related (i.e., apextrin and ATP synthase subunit 8), transcription factors, (i.e., pax C), oxidative phosphorylation (i.e., cytochrome oxidase subunit 1 and cytochrome P450), and apoptosis (i.e., caspase 8). Globally, the highest percentage of upregulated proteins corresponded to stress response (i.e., heat shock transcription factor and NADH ubiquinone) followed by cell signaling (i.e., minicollagen and alpha carbonic anhydrase), and cell cycle (i.e., calmodulin) with 83%, 75% and 66%, respectively.

With respect to downregulated protein functions, 100% appear to be involved in immunity (i.e., apextrin and ATP synthase subunit 8), 83% in UV-related and oxidative phosphorylation (i.e., green fluorescent protein and cytochrome oxidase subunit 1), cytoskeletal binding, transcription factors and apoptosis (i.e., actin, pax C and caspase) with 75% (Table 2, Fig. 5A, B, C and D).

RT-PCR for Acropora palmata

RT-PCR was used to validate the consistency of the five candidate genes between transcription and translation levels in *A. palmata* samples during the cool dry and hot wet seasons. A real-time dissociation curve confirmed the presence of a specific PCR product in all amplification reactions. The relative quantities of cytochrome P450, pax C, minicollagen, acropsin and caspase 8 genes are shown in Table 1. We examined three candidate housekeeping genes (HKG) (ribokinase, calcium-dependent protein-kinase and actin (DeSalvo et al. 2010)), however, the first two HKG were not found to be expressed in the sample *A. palmata.* Based on the results, actin was a suitable gene choice for normalizing target gene expression as determined by real-time RT-PCR. Actin showed a high level of expression with Threshold Cycle (C_T) value below 32 and it was selected for further evaluation of expression stability. The screening of the expression levels of five targeted genes for *A. palmata* revealed high expression for Pax C (C_T value 32.07), during the hot wet season in Enrique reef, while in acropsin (C_T value 34.43) and caspase 8 (C_T value 35.62) the expression levels were higher during the hot wet season in both reefs. Conversely, the expression of minicollagen and cytochrome P450 were respectfully downregulated to CT values of 15.04 and 10.85. The cytochrome P450 was uniquely expressed during the cool dry season in San Cristobal reef (Fig. 7).

Statistical analysis

A one-way analysis of variance (ANOVA) revealed that seasonal (cool dry and hot wet seasons) differences in the proteomic profile of *A. palmata* during 2014 and 2015 were significant ($p < 0.0014$). However, no significant differences were detected between 2014 ($p < 0.01$) and 2015 ($p < 0.05$) (with ± 95% confidence interval) (See Supplementary Material 2).

The qPCR data from all five genes, Pax C, minicollagen, cytochrome P450, acropsin 3 and caspase 8) was transformed and normalized with the CV analysis and Pairwise ΔCt method proposed by Hellemans et al. (2008). For accurate and robust normalization, three expressed reference genes were required (DeSalvo et al. 2010); in our case actin, ribokinase and calcium-dependent protein-kinase. The classic

delta- delta-Ct method was used with the reference gene actin as the most stable gene under the temperature variable during the two seasons. This model involves a calculation of ΔCt with stability values (M) and coefficient of variations (CV) lower than 1 and 50%, respectively. All the data analysis was developed using *t*- test with InfoStat statistical software (version 2014) (See Supplementary Material 3).

Discussion

The proteomic response of *A. palmata* to temperature changes during cool dry and hot wet seasons was significant with a high percentage of expressed proteins related to stress response. From these, 66% of the proteins were upregulated in Enrique reef and 27% in San Cristobal reef (Fig. 5), two reefs separated by a 3.5 km distance. Arbitrary fold change (FC) cut-offs of > 2 and significance p-values of < 0.02 lead data collection to look only at proteins which vary wildly amongst other proteins. Among the upregulated proteins figure NADH ubiquinone, glutathione S-transferase, cysteine, activin 1 and heat shock proteins (HSPs) which are known to be associated with survival recovery, metabolic cellular and stress processes. During September and October of 2014 and 2015, some of the warmest sea surface temperatures were recorded in La Parguera, Puerto Rico, therefore the upregulated proteins are consistent with the proteomic profile of an organism under cellular and oxidative stress likely caused by the increased ambient water temperatures.

Less than 33% of the proteins of *A. palmata* in both reefs (Table 2), such as beta tubulin, gelsolin, and myosin, were downregulated when compared with those expressed during the cool dry season. These proteins are regarded as key proteins known to interact closely with heat-shock proteins and immunophilins that play important roles in cellular repair and protective mechanisms during thermal stress in corals (Polato et al. 2010; Rosic et al. 2010; Tambutté et al. 2011; Jin et al. 2016). Downregulation of these proteins has been shown to activate oxidative metabolism and the capacity to generate ATP and NADPH, known to decrease the amount of glucose transporters in corals (Jones et al. 1998; Voolstra et al. 2009b; Rosic et al. 2014; Kaniewska et al. 2015).

Additionally, caspase 8 and ectonucleoside triphosphate diphosphohydrolase proteins, which are known to be mediators for proapoptotic pathways, were also decreased in their expression levels (1.40 and 2.71-fold, respectively). This downregulation suggests *A. palmata's* capacity to resist heat stress injury and avoid apoptosis by reducing the thermal stress signal and acclimatizing to ocean's warming and fluctuating environment (Gibbin et al. 2018; Howells et al. 2018; DeCarlo et al. 2019; Kubicek et al. 2019). On the other hand, acropsin 3, alpha q G protein and integrin beta proteins involved in photoreception processes, light-guided behaviors, immune and metabolic dysfunctions (Mason et al. 2012; Ricaurte et al. 2016) were slightly affected with a downregulation of 19%. This indicates that these three proteins can form functional photoreceptors and potentially play a role during light-guided behaviors and can play a unique role in changing the orientation of their polyps under light stress (Plaza et al. 2003; Roth et al. 2010; Harrison 2011; Hilton et al. 2012; Hemond et al. 2014; Iluz and Dubinsky 2015; Wuitchik et al. 2019) adapting the coral to these changes by the effects of light (Gates et al. 1992; Miller et al. 2007; DeSalvo et al. 2008). We suggest that under high temperatures, one of the mechanisms used by *A. palmata* to

maintain its vital functions such as calcification and respiration is reserving energy (Jones et al. 1998; Banaszak and Lesser 2009; Mayfield et al. 2011; Bove et al. 2020). Furthermore, protein such as apextrin and, ATP synthase subunit 8 were downregulated by 12%. This low percentage may reflect the possibility that some functions of *A. palmata* (defense response, carbon-concentrating mechanism development, growth, and energy metabolism) were not greatly compromised (Fig. 5A and C). ATPase subunit enzyme uses the energy from ATP hydrolysis to transport H + across biological membranes. It plays a universal conserved role in eukaryotic essential cellular functions such as the acidification of lysosomes and endosomes (Teixidó et al. 2020). In marine animals, it is known for specialized physiological functions participation in a widespread carbon-concentrating mechanism that promotes photosynthesis by *Symbiodinium* which are transferred to the coral (Falkowski et al. 1984; Muscatine et al. 1984., Tresguerres 2016). On the other hand, apextrins are known to be uniquely prevalent in the invertebrate phyla, especially in marine invertebrates. The C-terminal region of apextrins is very conserved and was designated as an apextrin C-terminal domain (Huang et al. 2014). Previous studies have shown that apextrin are involved in the embryonic development of hydra and coral (Miller et al. 2007) and in the defense response of marine organisms (David et al. 2005). Furthermore, it should be noted that proteins such as acropsin 3, alpha q G protein and beta integrin were downregulated in the inner-shelf reef Enrique, while upregulated in the mid-shelf reef San Cristobal. These proteins are known to have photoreception functions, suggesting that since *A. palmata* colonies grow in shallow water and are more exposed to higher levels of UV light as compared to other coral species, they may require a greater response from chaperone proteins, potentially photoprotective pigments such as green fluorescent proteins (GFP) and HSPs (Salih et al. 2000; Alieva et al. 2008; Scucchia et al. 2020). The GFP and ATP synthase F0 subunit 6 were respectively downregulated by 1.24/1.53 and 5.71/5.36 fold. These proteins are known to have diverse functions mainly playing a role in energy metabolism, including (but not limited to) protection from UV radiation acting as natural sunscreens to absorb and dissipate solar ultraviolet radiations (Mydlarz et al. 2010). This potentially serves the coral colony as a defense mechanism during the oxidative stress response through their ability to quench Reactive Oxygen Species (ROS) (Glynn 1993; Roth et al. 2012; Granados-Cifuentes et al. 2013). A common environmental factor underlying the stress response of *A. palmata* in these two coastal reefs was identified as the higher water temperatures during hot wet season (3.0 to 3.5ºC higher during September and October). The proteomic response of *A. palmata* was characterized by a higher protein expression of stress markers such as heat shock transcription factor and activin 1 protein, among others. The differences of fold-changes in proteins involved in the stress response found between the two reefs could be linked to the potential of *A. palmata* to cope with changing environments. Particularly in reef Enrique's with higher levels of expression of HSPs, indicating a differential vulnerability of *A. palmata* species under a climate change scenario.

Examples of key differentially expressed proteins, include transcription factors such as pax c (4.4/6.3-fold), HSP sp H16.2 (5.3/7.8-fold), glutathione S-transferase (1.3/17.0-fold), NADH oxidoreductase (4.5/4.7-fold) and activin 1 transferase (6.2/4.6-fold). The cysteine-rich protein was the only stress protein that was downregulated by 7.8/6.5-fold, which has been shown to assemble HSPs to stress out the algal endosymbionts to leave the coral host (DeSalvo et al. 2008; Weston et al. 2012). The

downregulation of cysteine-rich proteins in corals has been associated to a molecular protective mechanisms expressing HSPs to resist thermal stress. Moreover, by associating with stress-resistant symbionts, some coral species can acquire greater thermal tolerance, as it might be the case for *A. palmata* (Caraballo-Bolaños et al. 2020).

To summarize our discussion, we propose the model outlined in Fig. 7. This model encompasses the hot wet-induced *A. palmata* differentially expressed proteins further investigated by KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment and Protein-Protein Interaction Networks Functional Enrichment Analysis through STRING databaseversion 11.01 (Supplementary Material 5). The proteins with high confidence scores were categorized based on the Gene Ontology (GO). Our proposed model (Fig. 7) implies that when ultraviolet light intensifies, temperature increases causing an immediate physiological response in *A. palmata*, thereby affecting its overall metabolism, growth, reproduction, and increased susceptibility to diseases, bleaching and potential death (Takahashi and Murata 2008; Naviaux 2018; Miloslavich et al. 2010; Sweet and Bythell 2016). Consequently, oxidative stress releases large quantities of ROS produced across the ectoderm and endoderm of the coral (Starcevic et al. 2010; Maor-Landaw and Levy 2016) causing cytotoxic effects in neighboring cells. Among these effects, DNA damage and altered ribosomal and protein synthesis can be observed during thermal stress. The cytochrome upregulated P450 hemoproteins detected in San Cristobal reef during cool dry season might be involved in cell detoxification and protection from oxidative stress (Goldstone 2008; Voolstra et al. 2009) and therefore we hypothesize that this enzyme would be downregulated response to thermal stress in *A. palmata* as a typical well documented effect (Rosic et al. 2015).

On the other hand, important proteins such minicollagen and alpha carbonic anhidrase which were upregulated in both reefs during hot wet season have been recognized in the inner wall of the branch tips in the coral for defense (Palacios et al. 2014), and are involved in biomineralization and calcification processes (Wirshing and Baker 2014). These proteins were downregulated, putting forward another potential defense mechanism for *A. palmata* (Vidal-Dupiol et al. 2013). Since calmodulin has been shown to participate in responses to stress due to temperature and alteration of calcium homeostasis as well (Huang et al. 2018), its upregulation in both reefs during hot wet season suggests that calmodulin is a potential candidate marker for coral extracellular calcium influx (Rahman et al. 2014; Weston et al. 2015). Induction of several transcription factors such as pax C and BMP/2/4 in *A. palmata* indicates that regulation of cell-fate specification and tissue regionalization was affected (Chi and Epstein 2002). Interestingly, the downregulation of caspase 8, a pro-apoptotic marker, indicates that *A. palmata* is mediating an anti-apoptotic response. This suggests the possible involvement of caspase 8 in mechanisms that dictate the fate of the coral colony (i.e., death or recovery).

Consistent with the proteomics analysis, the expression changes identified by the 2D-GE LC-MS⁄ MS analysis were validated by RT-PCR analysis to assess the effect of cool dry and hot wet seasons on mRNA levels of selected identified proteins. Among the significantly upregulated genes specifically expressed during the hot wet season in the inner-shelf reef Enrique, included pax C, minicollagen, acropsin and caspase 8. These transcription factors are valuable markers for assessing early warning

signs of seasonal temperatures exceeding previous values as recently demonstrated in coral stress responses (Portune et al. 2010; Fuess et al. 2016).

Conclusion

This study represents the first attempt to observe changes of protein expression profiles in *A. palmata* in response to seasonal temperature fluctuations. Taken together, these results suggest that *A. palmata* is capable of activating metabolic reactions and synthesizing high-levels of stress response proteins. This balance the negative impact of elevated temperatures during the hot wet season as manifested in southwest Puerto Rico and subsequently may present an acclimation mechanism by which *A. palmata* responds during thermal stress episodes.

Our results highlight the predominant involvement of proteins associated with energy metabolism, stress-related proteins to maximize photosynthetic efficiency and acclimation responses under the prevailing seasonal variation in seawater temperature experienced by *A. palmata* in Puerto Rico.

The capacity of *A. palmata* to cope with 3.0 to 3.5°C fluctuations surpasses the upper thermal tolerance limits in seawater and may be indicative of the colonies' overall health status and their ability to successfully acclimate to new challenging environmental conditions. Generally, upregulation was the dominant trend for protein expression in *A. palmata*. Our findings illustrate the power of proteomics approaches to identify early-response protein markers and molecular processes involved in *A. palmata's* acclimation and lay the groundwork for future marine organisms-based analysis.

Declarations

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Figures

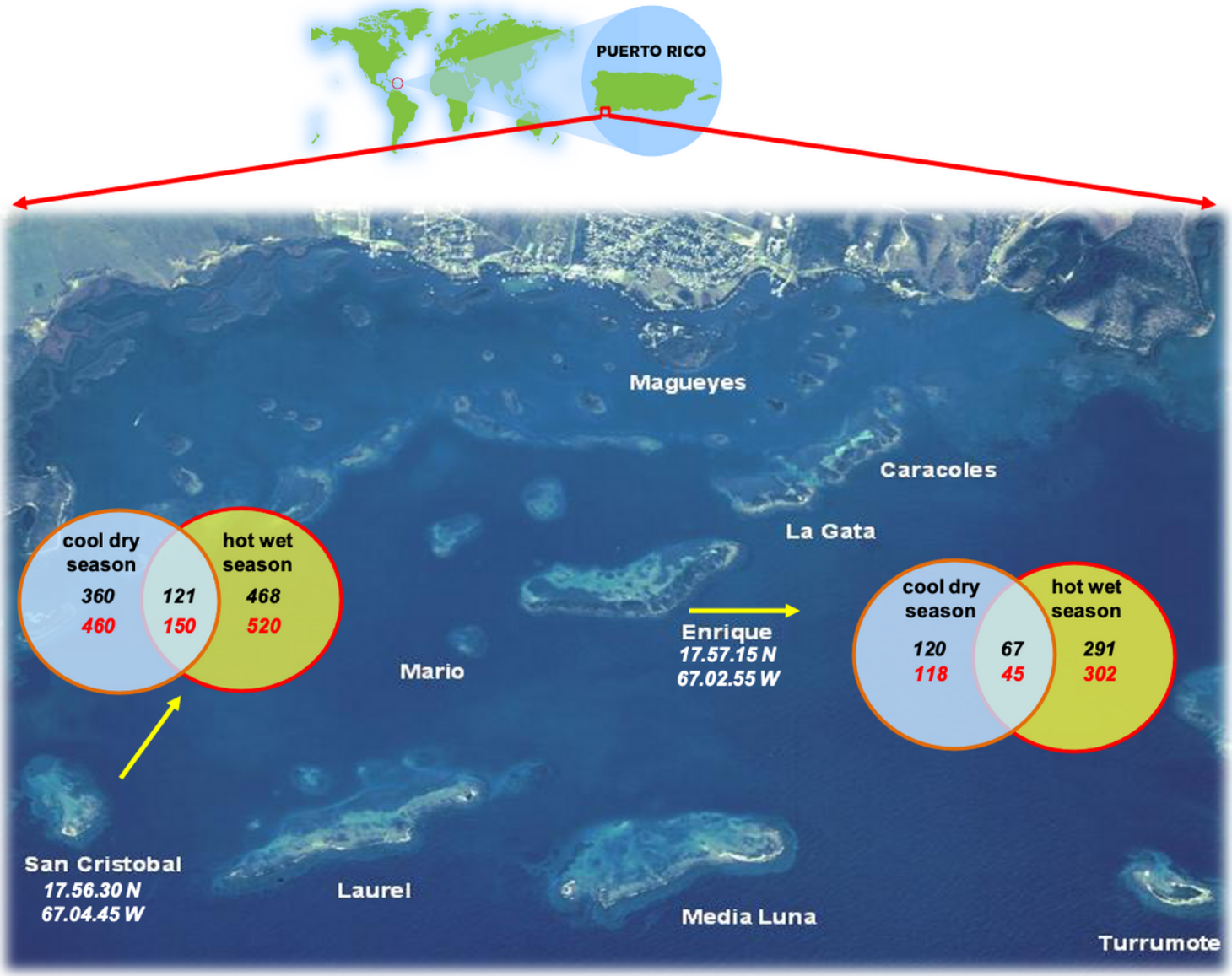

Figure 1

Map of La Parguera Natural Reserve showing the location of study sites along mid-shelf reef San Cristobal (A) and in the inner-shelf reef Enrique (B) with the corresponding Venn diagram indicating the differentially expressed proteins during 2014 (black numbers) 2015 (red numbers) for Acropora palmata during the cool dry and hot wet season in Puerto Rico.

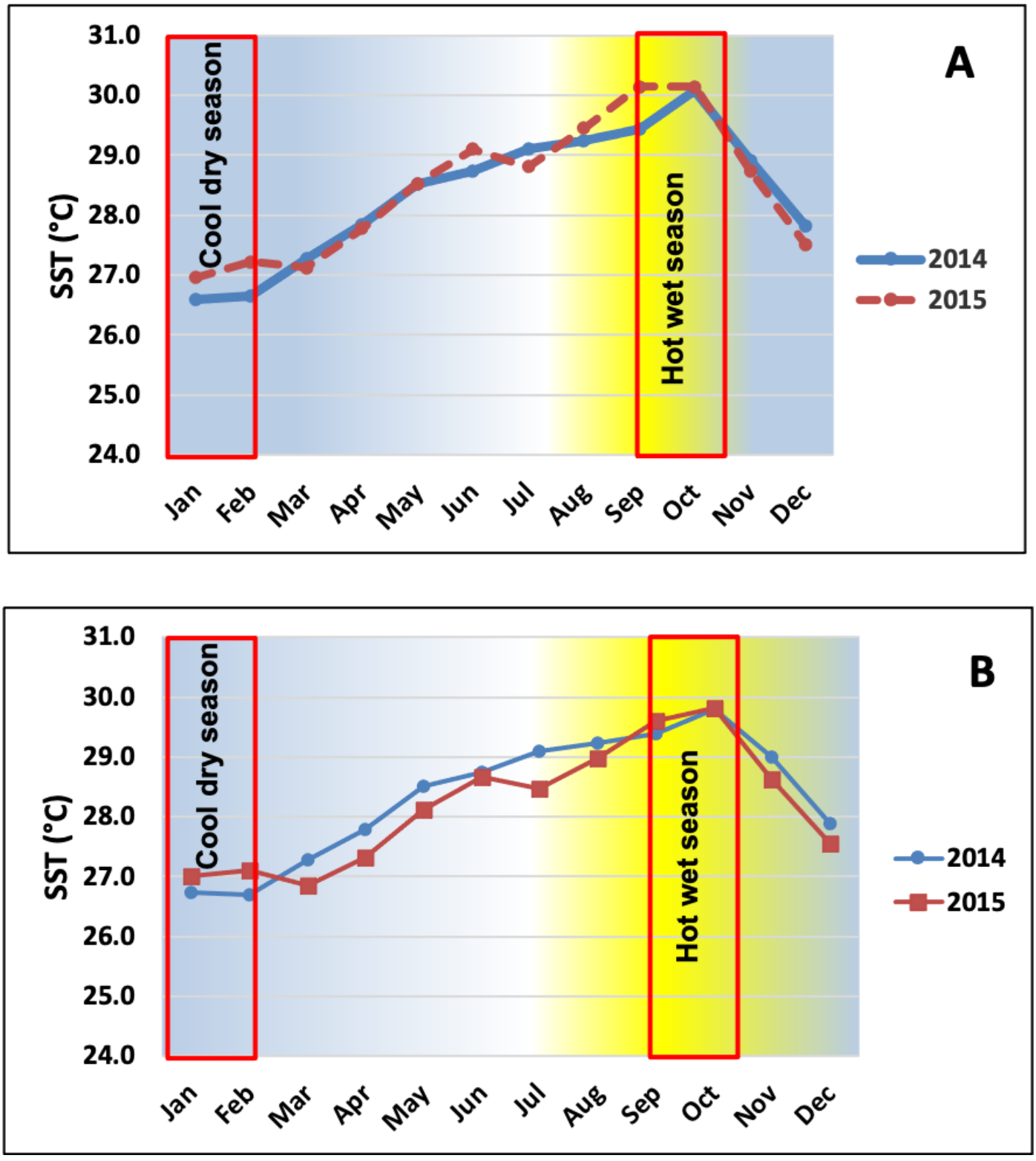

Figure 2

Monthly average sea surface temperatures (SST) during cool dry season (Jan-Feb) and during hot wet season (Sep-Oct) at the inner-shelf reef Enrique (A) and, in the mid-shelf reef San Cristobal (B), respectively in La Parguera, Puerto Rico during 2014 and 2015.

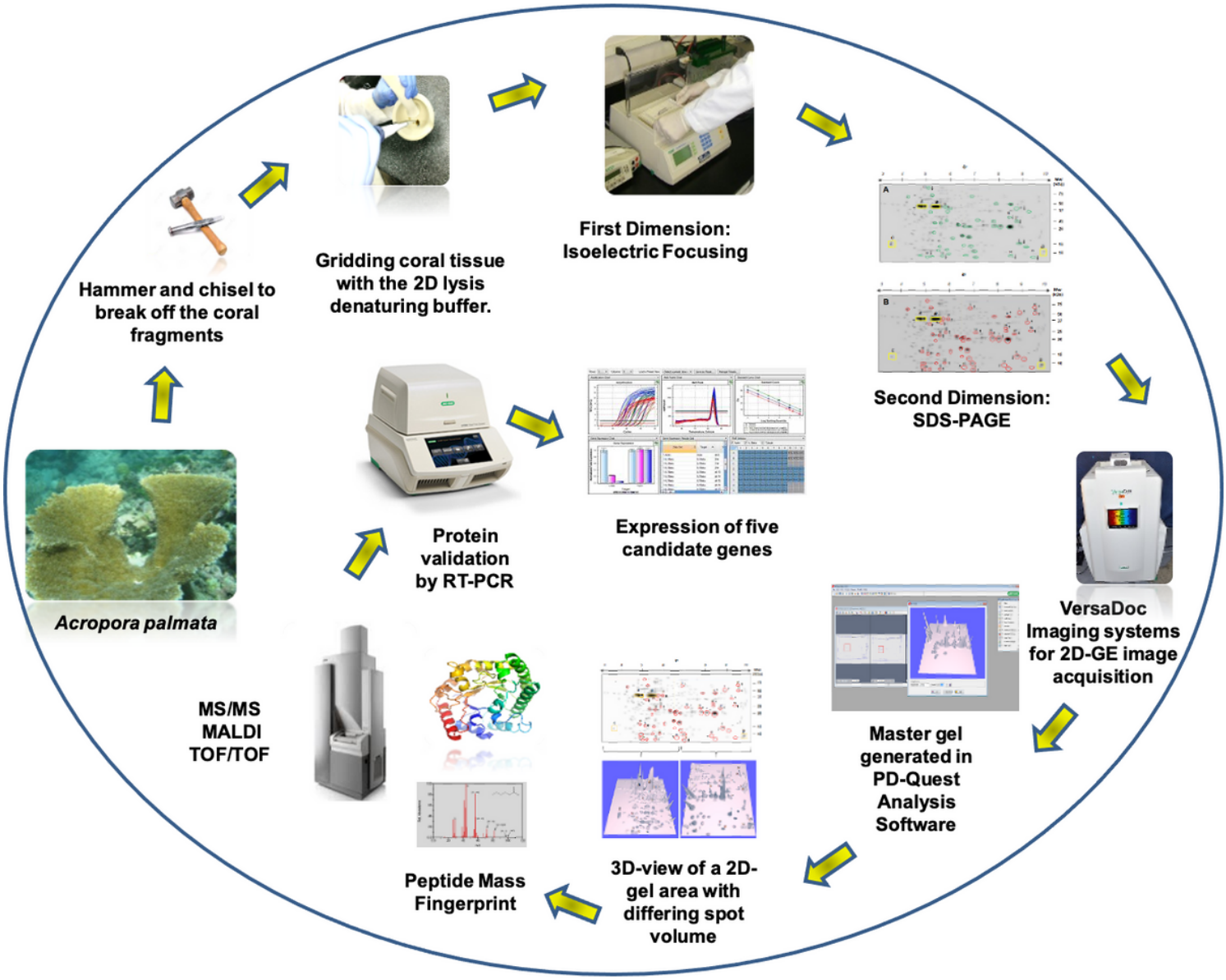

Figure 3

Schematic representation and workflow of proteomics analysis in Acropora palmata during the two seasons in 2014 and 2015.

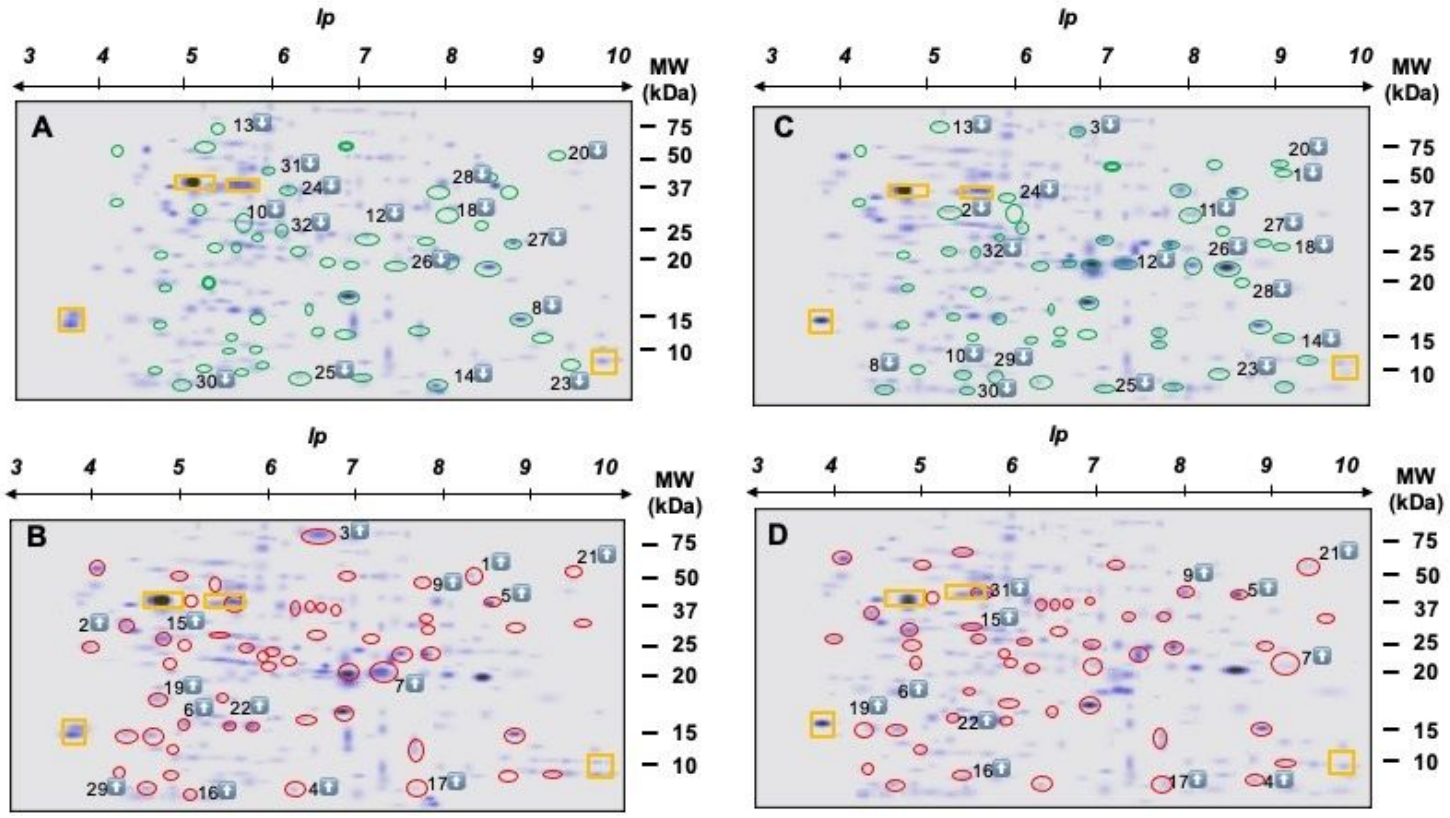

Figure 4

Two-dimensional gel electrophoresis showing differentially expressed proteins in Acropora palmata during cool dry season (A) and hot wet season (B) in the inner-shelf Enrique and cool dry season (C) and hot wet season (D) in the mid-shelf reef San Cristobal. The red and green circles with numbers and upwards and downwards pointing arrows on the 2D gels, indicate the proteins analyzed by MALDI-TOF-TOF (1 to 32). ⬆: Upregulated proteins, ⬇: Downregulated proteins. Yellow squares represent common proteins.

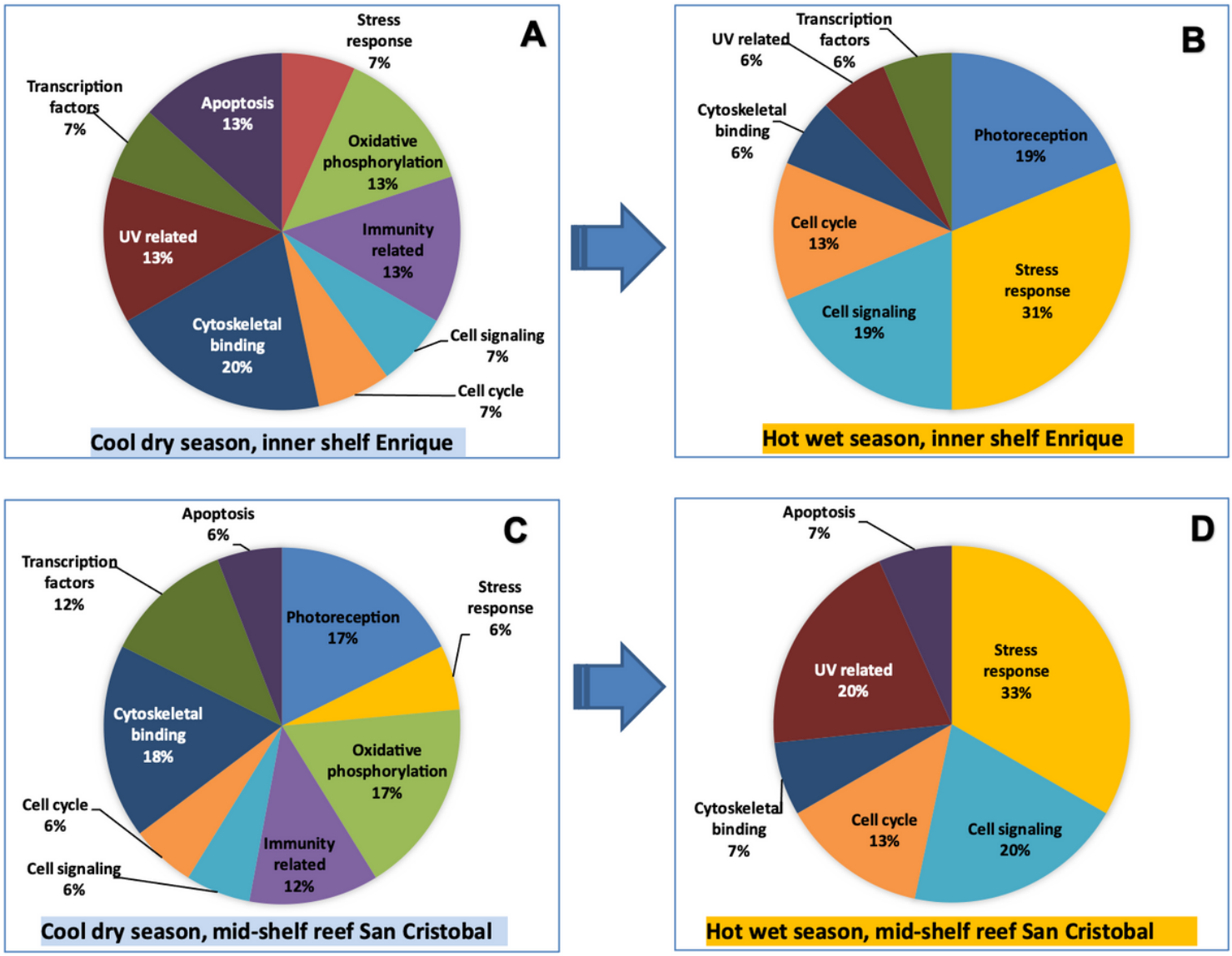

Figure 5

Pie representation of the proteins expressed according to their function in Acropora palmata. A. Cool dry season at the inner-shelf reef Enrique, B. Hot wet season at the inner-shelf reef Enrique, C. Cool dry season at the mid-shelf reef San Cristobal, D. Hot wet season at mid-shelf reef San Cristobal. The arrows represent the transition from one season to another.

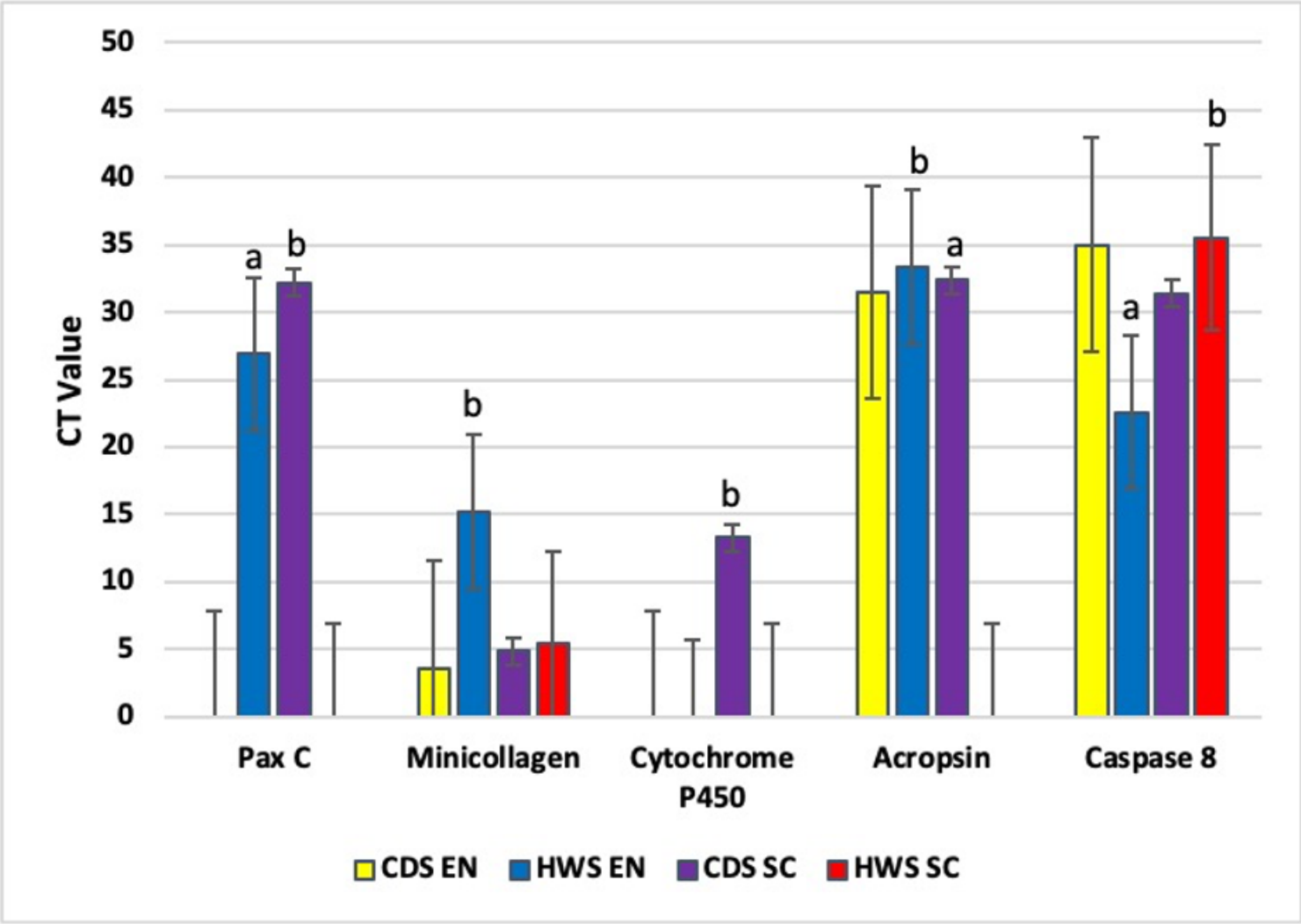

Figure 6

Evaluation of the expression for five candidate reference genes in Acropora palmata. Yellow bar: Cool dry season at the inner-shelf reef Enrique. Blue bar: Hot wet season at the inner-shelf reef Enrique. Violet bar: Cool dry season at the mid-shelf reef San Cristobal reef. Red bar: Hot wet season at the mid-shelf reef San Cristobal. “a” represents significantly up-regulated proteins ($P < 0.05$) and “b” represents significantly down-regulated proteins ($P < 0.05$).

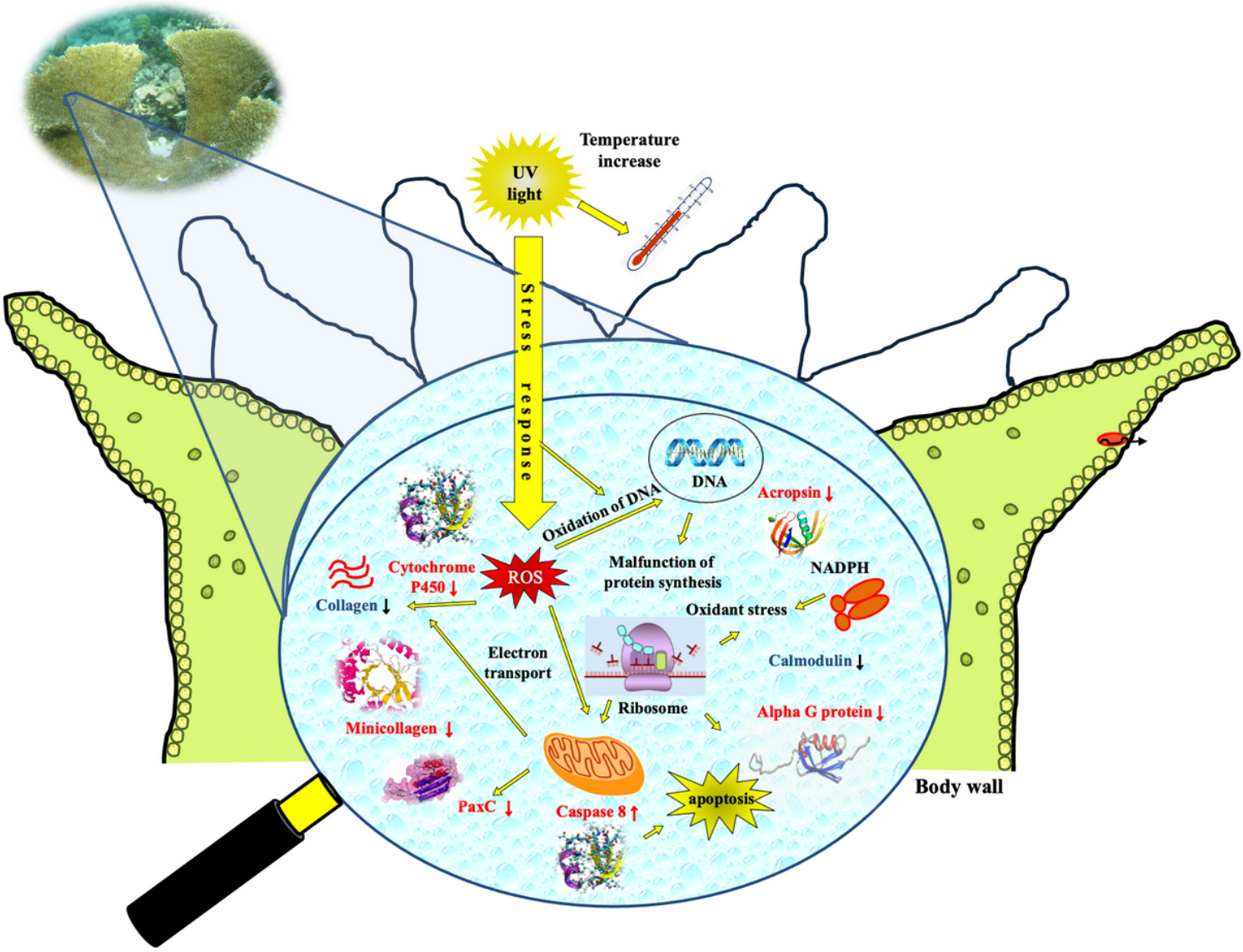

Figure 7

Diagrammatic representation of the metabolic pathway affected by the hot wet season in Acropora palmata. The gene expression levels of the overexpressed proteins highlighted in red and identified through a mass spectrometry-based proteomics approach were validated by RT-PCR.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.09.07.21.pdf
- Table2.2ndpaper9.7.21.pdf
- mergedSup.mat1.pdf
- Supmat.7.xlsx