Telomere dysfunction is associated to dark-induced coral bleaching in the reef coral Stylophora pistillata

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Abstract

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Abstract

Telomere DNA length is a complex trait controlled both by multiple loci and environmental factors. Even though the use of telomere DNA length measurement, as a method of assessing stress accumulation and predicting how this will influence survival, is currently being studied in numerous human cohort studies, the importance of telomere length for stress response in ecological studies remains at its infancy. Here, we investigated the telomere changes occurring in the symbiotic coral*Stylophora pistillata* that has experienced

a continuous darkness over six months. This stress condition led to the loss of its symbionts, as what is also observed when bleaching occurs in the field at a large-scale due to climate changes and anthropogenic activities, threatening the worldwide reef ecosystem. We found that the continuous darkness condition was associated with telomere DNA length shortening and a downregulation of the expression of the telomere-associated protein Pot2. These results pave the way for future studies on the role of telomere in coral stress response and the importance of telomere dysregulation in endangered coral species.

Introduction

Scleractinian corals are metazoans that successfully built complex ecosystems, the coral reefs, which are among the most important biodiversity hotspots of the planet. However, all over the world, coral reefs are under threat due to global climate change and anthropogenic activities (Hughes et al. 2018b). These stressors can cause imbalances in the community of endosymbiotic partners hosted by corals such as viruses, bacteria, fungi and most notably, members of the algal dinoflagellate family *Symbiodiniaceae.* The breakdown of this intricate symbiosis leads to the loss of the intracellular symbionts and/or their pigments, a process that is called bleaching (Downs et al. 2013; Sully et al. 2019; Downs et al. 2009; Danovaro et al. 2008; Kushmaro et al. 1996; Rosenberg et al. 2009). Corals can either recover from bleaching by acquiring new symbionts or die in case of prolonged or repeated stresses. Of note, bleaching can be triggered in the laboratory under different experimental set-up (increases in temperature or light intensity, continuous darkness, hyposalinity, chemical pollutants and pathogen infection) but if food is provided, the corals can survive for long time periods even though different physiological processes can be affected (body mass loss, stop of calcification, growth arrest and polyp mortality) (Lyndby et al. 2020; Gardner et al. 2017).

In order to mitigate coral loss, there is currently an urgent need to understand the mechanisms involved in the response of corals to stress and among them, the pathways maintaining genome stability are essential. An accurate response to DNA damages caused by environmental stressors (radiation, heat, pollutants, nutrient fluctuations...) as well as normal metabolic processes (such as replication, respiration and, in the case of symbiotic corals, photosynthetic activity leading to daily oxidative switches), requires efficient DNA damage checkpoints, DNA repair and survival pathways to maintain genome stability and health (López-Otín and Kroemer, 2021). Therefore, the efficiency of DNA repair mechanisms is one of the main regulators of stress resistance and longevity in many organisms (Ma et al.,2016; Tian et al., 2019). However, aside from anti-oxidant defenses (Furla et al., 2005) and UV DNA repair (Reef et al., 2009) and recent report on increased levels of phosphorylated H2AX (a marker of DNA damage response) (Rodriguez-Casariego et al., 2018) and Rad51 (a DNA double strand break repair protein) (Maor-Landaw and Levy, 2016) during heat stress, our knowledge on the role of genome maintenance pathways in environmental tolerance in corals remains limited.

Among the genome stability mechanisms, the chromosome ends, or telomeres, play a key role in stress response. Telomeres are composed of DNA repeats, (TTAGGG)n in most metazoans (Traut et al. 2007), bound by a protective protein complex, the shelterin, which protects the chromosome against unwanted DNA damage response (de Lange 2005). In humans, the shelterin complex is made of six proteins (TRF1, TRF2, TIN2, TPP1, POT1 and RAP1) (Giraud-Panis et al. 2013). TRF1 and TRF2 (Telomeric-repeat binding factor 1 and 2) directly bind the duplex telomeric DNA repeats, POT1 (Protection of Telomere 1) binds the single stranded telomeric DNA forming the 3' overhangs of chromosomal DNA, while TIN2 and TPP1 bridge the double and single stranded parts of telomeric DNA. In addition, RAP1 is associated with TRF2. The telomerase holoenzyme, comprising the reverse transcriptase subunit TERT and its RNA template, is able to maintain telomere length during DNA replication (Gilson and Geli 2007). Functional telomeres protect chromosomes against instability and senescence and are also involved in a wide range of important processes such as inflammation, immunity, energetic metabolism and mitochondrial integrity, stemness as well as cellular differentiation (Ye et al., 2014). Changes in telomere structure occur during development, stress response and aging. In general, exposure to unfavorable environments leads to accelerated telomere shortening (Young et al., 2017). In birds, telomere DNA length can predict key life-history traits like growth, reproduction and lifespan (Monaghan, 2014). Thus, it was proposed that change in telomere DNA length is

an adaptive strategy based on life-history regulation and environmental adaptation (Young 2018) and that telomere DNA length is a useful biomarker for past stress (Bateson, 2016).

Here we investigated the impact on telomere structure of continuous darkness stress (six months) in the model coral *Stylophora pistillata* (Esper, 1797). Notably, we observed that this stress condition leads to telomere DNA length shortening as well as a downregulation of the expression of a coral ortholog of a shelterin subunit. Since this stress is associated with the bleaching of the coral colony (Supplementary Figure 2), we discuss the possibility that the telomere response to continuous darkness is a consequence of the bleaching state.

Material and methods

Sampling

Colonies of the tropical coral *S. pistillata* were exposed to long-term darkness in an experimental aquarium setup for a long term exposure of six months. Briefly, coral fragments were kept in aquaria supplied with Mediterranean seawater (exchange rate of 70% per hour) at a salinity of 38 g liter^{-1}, temperature of 25degC, pH_T 7.94 +- 0.02 and for the control condition at an irradiance of 230 µmol photons m^{-2}s^{-1} on a 12:12 photoperiod. Both dark and control conditions corals were fed daily with frozen rotifers and twice a week with live artemia nauplii. The first experiment D1 was conducted from July 2018 to January 2019 were a part of the S1 colony was separated and put in dark condition, 6 branches of the bleached colony were sampled and 5 of the control one, the second experiment D2 was conducted from June 2019 to December 2019 were a different *S. pistillata* colony was divided in 2 part, each one kept in either of the two experimental condition, 4 branches from each colony part were sampled.

DNA extraction

DNA was extracted on the first 3-4 cm of a colony branch. All steps until cell lysis were done on ice. The fresh coral tissue was removed from the skeleton using the "air_brush" technique in 50mL of extraction buffer (NaCl 550mM, EDTA 0,2M (Tambutté et al., 2007)) in a Whirl-Pak bag (Nasco) under the hood, then centrifuged for 10 minutes at 5 000g (Beckman Coulter, Avanti J-E centrifuge, rotor JA-18) at 4°C. The tissue pellet was kept on ice to perform high molecular weight DNA extraction following the Midi Kit Cell and Blood culture (QIAGEN). Briefly, cell lysis was carried out for 2 hours at 50°C after adding 19 mL of G2 buffer (800 mM guanidine HCl ; 30 mM Tris·Cl, pH 8.0 ; 30 mM EDTA, pH 8.0 ; 5% Tween-20 ; 0,5% Triton X-100, QIAGEN) with 190 mL RNase A (ThermoFisher, ref : 12091039 , 20mg/mL) and 400 mL of protease (QIAGEN, 1 UA/mL) to the tissue pellets. Supernatant was poured in the anionic column after a 5 min centrifuge at 5 000g and 4°C, columns were washed following the kit instructions, DNA was eluted with 5mL of warm QF buffer (1.25 M NaCl ; 50 mM Tris·Cl, pH 8.5; 15% isopropanol, QIAGEN). Precipitation was performed adding 3.5mL of 2-propanol (Sigma), after mixing by inverting, tubes were stored overnight at 4°C. Two washing steps were performed adding 1mL of 70% ethanol (sigma) to DNA pellets after 30 min centrifuge at 15 000g and 4°C before adding 200 mL of TE buffer (Tris-Cl pH8 10mM, EDTA 1mM) for long-term storage. Pellets were resuspended at 50°C for 1 hour. To test DNA quality and quantity both Nanodrop (ThermoFisher) and agarose gel were used, respectively 1 mL of DNA and 1,5 mL of loading buffer (New England Biolabs, 6X) in a final volume of 10 mL were loaded in a 1.2% agarose gel run for 30 minutes at 100V in Tris-Borate-EDTA (TBE) 1X. Agarose gel was incubated for 10 minutes in 4 mg/mL ethidium bromide and DNA was revealed under UV (GelDoc Transilluminator, BioRad), the average of three measurements was used. A diluted aliquot was prepared for enzymatic digestion before storing the stock DNA at -20°C.

Telomere Restriction Fragment assay:

Dilution and DNA digestion

To digest non telomeric DNA sequences, we added 60U HinfI (R0155M, New England Biolabs) and 60U RsaI (R4374, Promega) to of 2,5 mg of DNA diluted in mqH2O and "CutSmart Buffer" 1X (New England

Biolabs) in a final volume of 40mL, left overnight at 37°C.

Southern Blot

We measured telomere length using the Telomeric Restriction Fragment assay (TRF) using the Southern blotting procedure (Herbert et al., 2003) digested DNA was loaded on a 1,2% agarose gel for a 3h30 run in TBE 0.5X at 9 volts/cm in a CHEF-DR-II (BioRad). The run timing makes it suitable for 2 runs a day. After a 5 min wash in mQ H2O agarose gel was stained in Ethidium bromide bath (4 mg/mL) under agitation for 30min and imaged the gel (Typhoon, GE Healthcare, Fluorescence, Method=Alexa Fluor 555, Laser=532nm, PMT=ch.1,700V, Resolution = 50 µm). Denaturation was done in (1 M NaCl ; 0.5 M NaOH) for two incubations of 20 min followed by a neutralization step with two 20 min incubation in 1M ammonium acetate. The DNA was transferred to a Hybond N^+membrane (GE Healthcare) overnight in SSC (20X). DNA was cross-linked on the membrane using the Ultraviolet Crosslinker (UVP) at 1200 x 100µ Joules. Membrane was gently washed in mQH_2O before storage at room temperature for (12h up to 48h) before 1h pre-hybridization in Denhardt's buffer (10X) at 40°C. We prepared stocks of Denhardt's 100X buffer (2% Ficoll 400, 2% Bovine Serum Albumine, 2% Polyvinylpyrrolidone) filtered (0.2µm) and stored at -20°C.

Probe synthesis

We prepared probe template 40uM stocks annealing two primers per probe. Probe targeting (TTAGGG) sequence will be called T2 and the one targeting (TTTAGGG) sequence will be referred to as T3. We mixed 25 of Primer F and 25 of Primer R (Primer **T2AG3_F** : GGGTTAGGGTTAGGGTTAGGGAAA and **T2AG3_R** : TTTCCCTAACCCTAA, Primer**T3AG3_F** : GGGTTTAGGGTTTAGGGTTTAGGGAAA and **T3AG3_R** : TTTCCCTAAACCCTAAA) with STE 5X buffer (Tris 50mM pH8, NaCl 250mM, EDTA 5mM) and heat it to 95°C to therefore gradually let it cool down to room temperature before being stored at -20°C. We mixed on ice 2.5 of dATP (10 mM) and 2.5 of dTTP (10mM, New England Biolabs, R0192) with 3of NEB 2 buffer (10X), 15of mQH2O, 5 of gamma-dCTP32 and 1 Klenow (3'–>5' exo-) (M0212S, NEB) incubated in a thermocycler (ThermoFisher Scientific) for a (30min 25°C, 5min 98°C, 5min 25°C) program. Then we purified the probes following the ProbeQuant G50-micro columns (GE, Healthcare) instructions. Probes were denatured at 95°C for 5 min and used or stored at -20°C.

Hybridization

Membranes were pr hybridized for 1h at 40°C in hybridization Denhardt's buffer (10X). We added 5µL of denatured T3 probes to 15mL of hybridization buffer for an overnight hybridization at 40°C. Washing steps included a 2x20min wash in 2X SSC, a 30min step in (2X SSC, SDS 0,2%) and a final H2O washing step. Membrane was carefully wrapped in plastic sarran film and applied to a phosphor-screen (brand) for 60h. We imaged the phospho-screen on the Typhoon (GE Healthcare, Phosphorimaging, Method=[Phosphor], Laser=635nm, PMT=ch.1,1000V, Resolution = 50 µm), the screen was flashed (brand of device). Membrane was stripped with NaOH 0,4M for 30min at 42°C, then for 30min in (SDS 1%, SSC 0.1X, Tris-HCl pH7.4 1M) to remove the T3 probe. To control the stripping, membrane we exposed the membrane to a flashed phosphor-screen for 12h. The screen was imaged as described above, in the absence of signal the membrane was pre hybridized for 1h and hybridized overnight at 45°C with the T2 probe, otherwise the membrane was stripped again until no signal was imaged after 12h of exposure. Washing steps and imaging were carried out as described above with a 24h exposure of the phosphor-screen.

Image analysis

Telomere Restriction Fragment (TRF) images signal was extracted using ImageQuant (GE Healthcare) 1D gel analysis mode and manual lane creation. Ladders signal intensity were extracted from Ethidium bromide gel images, setting the lane upper limit at the gel wells bottom. Pixel position of ladder peaks were manually reported. Host and symbiont telomere signals were extracted from the phospho screen, exposed to radioactive labelled membrane, images setting sample lane upper limit at the top of the membrane. Efficiency of stripping step between the two probes hybridization was assessed imaging phospho screens overnight exposed to stripped membranes.

Telomere Length measurements

Single lane intensity files were fused in RStudio. Ladder peaks exact position was extracted in R, searching for the maximum intensity in a 10-pixel perimeter around the manually reported peaks. Fitted linear model coefficients (a,b) of log2 ladder size (kb) against peak pixel position were calculated for high molecular weight (48.5-15kb) and low molecular weight (10-1kb) using the *lm* function of "stats" R package. Coefficients were used to transform samples intensity scale from pixel to base pair (bp) () in Excel using the high molecular weight coefficient for the upper part and the low molecular coefficient for the lower one. Depending on their position on the membrane samples were divided in left, right and middle to be scaled to the closest ladder. Intensity signals were imported in R using the *read_excel* from "readxl" package, background correction was automatically computed to level the signal by subtracting to each position the minimal intensity. Intensity was normalized by the size to avoid probe number hybridization bias, intensity above 2 kb were discarded to avoid genomic noise, interstitial telomeric sequence noise and normalization bias (<1kb). Ponderate telomere mean was calculated as in (Li and de Lange 2003), median, quartiles, intensity signal, smoothed signal and telomere measurements were plotted for each sample using the *ggplot*function from "ggplot2" package and measurements were saved as*csv* files.

Transcriptome

RNA extraction

Coral pieces of approximately 2 cm were sampled just below the coral piece dedicated to DNA extraction, at least 4 cm from the apex of the branch. Samples were flash frozen in liquid nitrogen in WhirlPack bags, and then crushed in small pieces with a press and fragments were put in 600uL of lysis buffer RLT in PowerBeads Glass 0.1mm (Qiagen), samples were lysed in Bead Beating instrument for 4 minutes with 30 cycles/s. Samples were spin at max speed for 3 minutes and the 600uL of supernatant was transferred to a fresh tube. Add 600uL (1V) of cold EtOH 70% to the column (Qiagen, RNeasy Mini Kit), gently mixed by pipetting, immediately apply 600uL on the column and centrifuge 15s at 8 000g (10 000 rpm), discard the flow-through, repeat this step. Add 350 uL of RWI buffer to the column and centrifuge 15s at 8 000g (10 000 rpm), discard the flow-through. Take a DNAse, Rnase free, (Qiagen, 79254) stored at -20°C, add 70ul of buffer RDD 4°C and apply to the column. Leave the column at RT for 15min before adding 350 uL of RWI buffer and centrifuge 15s at 8 000g (10 000 rpm), discard the flow-through and add 500uL of RPE buffer, repeat the centrifugation, add again 500uL of RPE buffer and make a 2min at 8 000g (10 000 rpm) centrifuge before discarding the flow-through and putting the column in a clean 1.5ml tube. Add 30ul of RNase free water to the column and centrifuge for 1min at 8 000g (10 000 rpm), keep the tube on ice for further experiment and store at -80°C. RNA quantity was measured on a microplate spectrophotometer Epoch (Bioteck) and the quality was assessed by running a 1% agarose gel labelled with Ethidium Bromide and imaged under UV-exposure in a Fusion Fx7.

RNA sequencing

Paired-end sequencing (read length: 2x150bp) was performed by NovoGene company using HiSeq sequencer, raw data are available with accession number GSE171268 (*https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE171268*). Three biological replicates per condition were performed with roughly 45 million reads per sample. Reads were trimmed using fastp software (Chen et al., 2018). Reads were mapped on the *S.pistillata*transcriptome from NCBI (GCF_002571385.1) using Salmon software (v0.11.4) (Patro et al., 2017). The differential genes expression analysis was performed using DESeq2 R package (Love et al., 2014). A cutoff of 0.05 was applied on adjusted p-values, obtained using Benjamini and Hochberg correction.

Ontology and pathway analysis

Gene Ontology (GO) analysis was performed using the "topGO" R package (Alexa et al., 2006). For the cnidarian analysis, all GO terms identified in *S.pistillata* were used as background. Ingenuity Pathway Analysis (Krämer et al. 2014) (QIAGEN,*https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis*)

was performed on human ortholog genes. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed using "gage" and "KEGGREST" R packages (Kanehisa, 2000). The human ortholog genes associated with the *S.pistillata* log2FoldChange values were used to conduct the analysis. Human orthologs were identified using the protein sequences. Blastp (Altschul et al. 1990) on UniProtKB/Swiss-Prot database restricted to *Homo sapiens* species was performed with a 1e-5 e-value threshold. For each protein, the best hit was selected, based on e-value and bit-score.

Phylogeny

Ortholog sequences of *S.pistillata* shelterin proteins were curated using PSI-BLAST (Altschul et al, 1997) on the non-redundant (Nr) protein database from the NCBI. The search was restricted to Metazoa group. The retrieved sequences were aligned using MAFFT v7.310 with L-INS-I algorithm (Katoh and Standley, 2013). The multiple alignment was trimmed with TrimAl v1.4 with the *gt 0.6* option (Capella-Gutierrez et al., 2009). Then the best substitution matrix was assessed using ModelTest (Darriba et al., 2020). Finally, the trees were built using raxmlGUI 2.0, a graphical interface for the implementation of RAxML Next Generation and the transfer bootstrap expectation branch support (Edler et al., 2020). Tree visualization was done with iToL v6.1 (Letunic and Bork, 2019). Multiple alignment for POT1 and POT2 were wrapped using Alignment Annotator (Gille et al., 2014).

Quantitative Polymerase Chain Reaction (PCR)

Primers were designed using PrimerBlast (Ye et al., 2012) and sequences are available in (Supplementary Table 3). Reverse transcription was carried for 1ug of RNA samples using the Applied Biosystems reverse transcription kit for 1h at 37°C followed by 5min of denaturation at 95°C. qPCRs were made on cDNAs obtained using ROCHE's Fast universal Sybr Greenmaster (ROX) on a StepOne plus thermocycler (Applied Biosystems). Each sample was triplicated on the 96 wells PCR plate containing the interest gene primers and the two control genes. In addition to the samples from the RNAseq, samples from the same experiment were added to the qPCR, two additional control samples (S1n2 and S1n4) as well as two treated samples (S1Bn2 and S1Bn6).

Statistics

Telomere length measurements were averaged from two independent Telomeric Restriction Fragment assay, homoscedasticity and normal distribution were tested on R using (*fligner.test* and*shapiro.test* functions) and significant differences was calculated using the t_test from the "rstatix" packages with a Bonferroni pvalue adjustement. Significant differences are reported as stars ((ns) non significant, (*) $P<0.05$, (**) $P<0.01$, (***) $P<0.001$.

Results

Putative telomere proteins encoded in the *Stylophora pistillata* genome

To characterize the structure of coral telomeres, we searched in the*S. pistillata* host genome (Voolstra *et al.* 2017) for genes encoding putative shelterin subunits as well as the telomerase catalytic subunit TERT. We identified orthologs of four human shelterin subunits (*Rap1, Trf1/2, Pot1, Tpp1*) and of *Tert* (Figure 1A-B-C-D-E). The coral genome contains two human *Pot1* paralogs, similarly to mouse and *Caenorhabditis elegans elegans* (Raices*et al.* 2008), and only one *Trf* , in agreement with the duplication of the *Trf* ancestor in *Trf1* and *Trf2*that occurred at the base of the chordate lineage (Poulet *et al.*2012) (Figure 1B). The coral Pot1 protein shares a similar structure with the human Pot1, containing two Oligonucleotide/Oligosaccharide-Binding (OB) fold domains located in the N-terminal region and a characteristic split OB fold in the C-terminus (Figure1-G, Supplementary Figure 1). The second ortholog of Pot, named Pot2, contains two OB folds skewed to the C-terminal part of the protein. Therefore, the protein composition of coral telomeres is likely to share many similarities to those of other animals (Figure 1F).

Telomere DNA length shortening in bleached samples

Southern blotting is considered the gold standard technique for telomere DNA length measurement (Aubert, Hills and Lansdorp, 2012). To measure the telomere DNA length of the coral host and its symbiont simultaneously, we adapted the Southern blotting procedure to coral tissue samples and performed sequential hybridizations, first with a probe recognizing the symbiont telomere sequence (TTTAGGG)n and then with a probe recognizing the animal host telomere sequence (TTAGGG)n. Consistent with previous publications on other coral species (Sinclair et al., 2007; Tsuta et al. 2014; Tsuta and Hidaka 2013; Zielke and Bodnar, 2010), colonies of *S. pistillata* revealed terminal DNA fragments hybridizing with a host telomere DNA probe of a mean varying from 3.3 kb to 5.7 kb and with the symbionts telomere DNA probe of a mean varying from 3kb to 6.2 kb (Figure 2A).

We induced stress by putting coral colonies in constant darkness (Douglas, A.E., 2003), in two duplicated experiments referred to as D1 and D2 (Supplementary Figure 2). In both experiments *S. pistillata* colonies were fully bleached after long-term treatment (six months) (Supplementary Figure 2). The telomere analysis was performed on branches of the same colony cut in two and maintained either in control or dark conditions for six months: for D1 (respectively D2), six (respectively four) branches from the bleached half colony and three (respectively two) branches from the control half colony (Figure 2A) were successfully measured twice in Telomere Restriction Fragment assay. The host telomere DNA length was measured reporting the smear mean, median, first quartile (Q1), third quartile (Q3) and interquartile distance (IQ) (Figure 2B, Supplementary Table 1). In both experiments bleached colonies were experiencing shorter telomeres with significantly shorter telomere DNA length mean, median, IQ and Q3 in the host and an absence of signal for the symbionts due to bleached state. However, Q1 measurements of the host telomere DNA length were not significantly different from those of control samples. Thus, the stress triggered by a six month dark incubation of *S. pistillata* shifted the host telomere DNA length distribution towards less long telomeres (Q3) without increasing the short telomeres (Q1) proportion.

Transcriptome profiling of dark-induced bleached *S. pistillata*

To unravel a telomere transcriptomic signature of stressed samples, we performed RNA-seq profiling of three control and three bleached branches from the D1 experiment. We identified 862 Differentially Expressed Genes (DEG) (adj. $p<0.05$), including 602 downregulated and 260 upregulated genes (Figure 3A and Supplementary Table 2). In order to identify the DEGs that could be directly controlled by the dark condition, we compared the transcriptomic profile of bleached samples to a previous transcriptome study performed on *S. pistillata*colonies during day and night cycles (Ottaviani et al. 2020) (Figure 3B). We found 31 genes in common and differentially expressed in the same direction between the two sets of DEGs (Figure 3B). We considered that the expression of these genes was controlled by the dark condition rather than the stress triggered by a long period in darkness and leading to a bleached state.

Using the telomere-related interactome reported in the Uniprot database, we identified among the DEGs nine genes that could be involved in telomere metabolism in the stressed samples (Figure 3C). One of the two upregulated genes (*Gnmt*) was predicted to be Glycine-N methyltransferase involved in DNA methylation, suggesting that the telomere changes triggered by the continuous darkness stress are associated with epigenetic changes. The second upregulated gene (*Trpc5*) is part of a ionic transporter family. Among the downregulated genes, five are known to be involved in cellular growth and tissue remodeling (*Cenpf, Plk1, Profilin-2, Mapk6* and*Plat*) suggesting a link between the telomere shortening occurring in stressed colonies and their reduced growth rate. We also found that*Pot2* , one of the two protections of telomere shelterin of*S. pistillata* , was significantly downregulated in bleached samples (Figure 3C). We confirmed, for a subset of genes (seven genes), their significantly expression changes by quantitative PCR analysis both in the samples used for RNA-seq and in biological duplicates using two reference genes with constant expression in control and stressed condition (*Trpc2* and *Pot1*) (Figure 3D, Supplementary Table 2 and 3). We confirmed a trend toward downregulation of *Pot2* that is not significant due to a single control branch (S1n4) that was expressing a very low level of *Pot2* (Supplementary Table 3).

The most significantly enriched ($p<0.01$) categories of gene ontology terms (GO terms) among the DEGs were related to metabolism, protein homeostasis, biosynthesis and oxidative stress (GO:0006979, GO:0055114)

(Figure 4A). When compared with heat induced bleaching transcriptomic studies done on various coral species (Figure 4B-C), we found three common GO terms in the Biological process that are related to growth, proteolysis and macromolecule catabolic process (Figure 4B-C and Supplementary Table 4) (Pinzón, 2015, Seneca and Palumbi. 2015, Traylor-Knowles et al., 2017, Zhou et al., 2017, Li et al., 2021).

Discussion

This study reveals that a reef coral experiencing a prolonged darkness leading to symbiosis disruption and bleaching exhibits signs of telomere dysfunctions. Notably a shortening of its mean telomere DNA length and a decreased proportion of long telomeres as well as the downregulation of the *Pot2* gene encoding a putative subunit of the telomere protective shelterin complex in coral. This finding is a new example of the intimate link between stress response and telomere dysfunction. In the context of the massive coral bleaching events due to the extreme rise of sea water temperatures, these results suggest that long term symbiosis disruption can affect host telomere state and should be taken into account to evaluate the fitness of coral reef survivors.

The enrichment of GO terms linked to oxidative stress response could reflect an increase in the production of reactive oxygen species (ROS), which are likely candidates to cause the observed telomere DNA shortening (Barnes et al., 2019). An increased production of ROS is also the cause of heat-induced bleaching in the wild suggesting a potential telomere dysfunction due to oxidative stress in heat-induced bleaching as what we observed in dark-induced bleaching. Since oxidative stress can be both the cause and the consequence of dysregulated telomeres (Jacome-Burbano and Gilson, 2020), bleaching could, through an increased oxidative environment, create a positive loop accelerating telomere dysfunction and favoring diseases. If oxidative stress is well documented to be a major cause of symbiosis breakdown due to an increase in photosynthetic activity (Lesser, 1997, Dias et al., 2019), the cause of the oxidative stress observed here after six months of darkness is not known. It could result from the maintenance of an oxidative stress response responsible for the initial symbiont loss and/or from a continuous hypoxic condition that can increase ROS production (Lewis et al., 2012).

Like in rodents and the nematode *C. elegans* , the coral genome contains two genes coding for human POT1 orthologs. Interestingly, the second coral *Pot* gene (named here *Pot2*) seems to have arisen after the divergence of Cnidarian organisms since *H. vulgaris* exhibits only one *Pot1* ortholog. In mammals, Pot1 forms a heterodimeric complex with Tpp1 and has a crucial role in regulating the resection of the telomere DNA overhang and in recruiting telomerase at telomeres (Nandakumar et al., 2012). The host telomere DNA length shortening observed in bleached samples could be explained by the downregulation of *Pot2* that would fail to recruit telomerase at long telomeres resulting in an overall decrease in telomere length after six months. Interestingly in another invertebrate that exhibits two Pot proteins, *C. elegans* telomeres harbor both 5' C- and 3' G-rich overhangs respectively bound by Pot2 and Pot1 (Raices et al., 2008). Since the 5' C-strand overhang can be involved in telomere elongation by recombination (Zhang et al., 2019), it is possible that a function of Pot2 in *C. elegans* is to regulate telomere elongation by recombination. To investigate whether corals exhibit both types of overhangs and how they are bound by the two Pot proteins could give interesting insights into the mechanisms of telomere DNA regulation in coral.

There are several limits to the conclusion that can be drawn from these results in terms of mechanisms of coral bleaching. First, the bleaching state studied here was triggered by continuous darkness, which is not a classical stressor acting in the wild (Sully et al., 2019; Hughes et al., 2018a). Second, the telomere and transcriptomic response could be more due to long term darkness than to the bleaching *per se* . Third, the bleached corals were kept alive by external feeding, and the shift from a partial to a complete heterotrophic state modified the coral metabolism and could be responsible for the observed telomere shortening.

The impact of long term darkness was visible in 31 DEGs previously identified in a study on *S. pistillata* looking at the day-night transcriptome (Figure 3B). When compared with temperature induced bleaching GO

term enrichment from studies in several coral species (*Acropora aculeus* , *Acropora hyacinthus* , *Pocillopora damicornis, Orbicella faveolata*), it appears that the common ones were first mostly linked to metabolism and second more numerous in the study using the closely related *Pocillopora damicornis* (Li et al., 2021). It would be of interest to test whether the combined effect of temperature and holobiont disruption can have a short term effect on telomere length regulation. It is important to point out that even under a constant feeding regime, bleached corals experienced a delay in growth, an impaired function found in enriched GO terms of different bleaching studies. Shallow corals have co-evolved with their symbionts and this relationship became mandatory as corals can't properly live without them.

Bleaching recovery is dependent on heat-sensitive to heat-tolerant symbiont turnover, showing the importance of the interplay between symbiont physiology and coral metabolism in survival (Claar et al., 2020). Thus, it would be of interest to investigate whether different symbionts association can lead to different telomere length regulation upon stress.

Nevertheless, the association between telomere DNA shortening,*Pot2* downregulation and oxidative stress response in an experimentally controlled darkness situation contributes to our understanding of the telomere changes occurring in coral that have experienced a stress and to the predictions of the impacts of climate change to telomere homeostasis and genome maintenance. Our results suggest that after a bleaching event, the telomere shortening that can persist with time, will affect the long-term health of coral reefs with a higher sensitivity to diseases. Moreover, the inheritability of parental stress bleaching experiences to offspring (Puisay et al., 2020) could be conveyed by telomere dysfunction and impact several generations among the surviving individuals.

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Data Accessibility Statement

The RNAseq raw data are available with accession number GSE171268 (*https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE171268*).

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Figure legends

Figure 1 : *Stylophora pistillata* shelterin proteins and telomerase phylogenetic trees. Phylogenetic trees based on protein sequences looking for human orthologs of *S. pistillata* shelterin subunits.(A) RAP1 phylogenetic tree supports the presence of a conserved protein between cnidarians (B) TRF phylogenetic tree supports the presence of only one TRF shelterin in cnidarians and two in vertebrates (TRF1 and TRF2) (C) TERT phylogenetic tree supports the presence of the telomerase holoenzyme subunit in cnidarians conserved protein between cnidarians (D) POT1 phylogenetic tree supports the presence of 2 POT1 shelterin in corals (*S. pistillata* , *A. digitifera* ,*P. damicornis*) such as in *C. elegans* (POT1 and POT2) (E) TPP1phylogenetic tree supports the presence of a conserved protein between cnidarians (F) Scheme of *Homo sapiens* shelterin complex and telomerase subunits and of *Stylophora psitillata* shelterin and telomerase subunit found in this work. (G) Localization of conserved domains (OB fold and Pot1-Cterminal domain) in human Pot1 and *S. pistillata* Pot1 and Pot2.

Figure 2 : *Stylophora pistillata* dark induced bleaching telomere length measured by Telomeric Restriction Fragment assay. (A) Telomeric Restriction Fragment assay from D1 experiment, left panel is showing the membrane labelled with the (TTAGGG)n coral DNA telomeric sequence complementary probe and right panel is the membrane labelled with the radioactive (TTTAGGG)n symbionts DNA telomeric sequence complementary probe. 5 control branches and 6 bleached branches are displayed. Bleached branches after 6 months of darkness exposure exhibits a shorter telomeric smear signal with the (TTAGGG)n coral telomere probe labelling and no signal with the (TTTAGGG)n symbiont telomere probe (B) Boxplot of (TTAGGG)n coral telomere smear measurements in kilobases (kb), IQ (interquartile distance between first

quartile Q1 and third quartile Q3), Mean telomere length and median telomere length between bleached and control samples from experiment D1 (circles) and D2 (triangles), TL IQ, Mean, Median and Q3 was significantly shorter in bleached conditions (respectively $p$value=0.0338,0.0338,0.498 and 0.0338) except for the Q1 that was not significantly different between the two conditions ($p$value=0.114).

Figure 3 : *Stylophora pistillata* bleaching RNA sequencing results. (A) Volcano plot of significantly differentially expressed genes in bleached condition (pvalue<0.05) of the RNA sequencing of 3 branches (n=nubbins) of control S. *pistillata*colony S1 and dark induced bleached colony (S1B). (B) Common differentially expressed gene in bleached transcriptome (left) and in night and day transcriptome (right) from (Ottaviani et al., 2020), log2(fold change) is displayed with colors (pink for negatively DEG and green for positively DEG). (C) Significant differential expression expressed in Log2(FoldChange) of telomere related genes (Gene names) in bleached condition ($p < 0.05$). (D) Fold change log of differentially expressed genes (Apod, CENPF, Profilin2, MAPK6, Pot2, Plk1, Ddx21, Trpc5, Gnmt, PXDN) performed by quantitative PCR using two different control genes (Pot1 and Trpc2) and by RNA sequencing. Statistics were performed using student tests on log results of Pfaffl equation (Supplementary table 3).

Figure 4 : Gene Ontology enrichment in bleached coral samples.(A) Biological process Go-term significant enrichment in dark induced bleached samples. (B) Venn diagramm of Go-terms compared with previous transcriptomic studies performed on heat induced bleaching samples. Three biological process Go-terms of our study were found in one of the four others as well as 1 cellular component and 1 molecular function one. (C) Common Go-terms between our study and the investigated transcriptomic studies. We included the study from (Zhou et al., 2017) but due to a total absence of common Go-terms this crossing was not displayed.