REPORT

Signaling pathways of heat- and hypersalinity-induced polyp bailout in *Pocillopora acuta*

Fabian Gösser¹ · Arne Raulf² · Axel Mosig² · Ralph Tollrian¹ · Maximilian Schweinsberg¹

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Abstract Polyp bailout is a drastic response to acute stress where coral coloniality breaks down and polyps detach. We induced polyp bailout in *Pocillopora acuta* with heat stress and tested for differential gene expression using RNAseq and a qPCR assay. Furthermore, we induced polyp bailout with hypersalinity and compared the results to identify stressor-independent signals and pathways active during polyp bailout. Both stressors led to the onset of polyp bailout and the detachment of vital polyps. We observed activation of microbe-associated molecular pattern receptors and downstream signaling pathways of the innate immune system. Further, we detected growth factors and genes active during Wnt-signaling potentially contributing to wound healing, regeneration, and proliferation. Upregulation of several genes encoding for matrix metalloproteinases and the fibroblast growth factor signaling pathway are the most likely involved in the remodeling of the extracellular matrix, as well as in the detachment of polyps from the calcareous skeleton during polyp bailout. Expression of genes of interest in our qPCR assay of vital polyps from our heat-stress experiment, showed a trend for a normalization of gene expression after polyp bailout. Our results provide new insights into the signaling cascades leading to the observed physiological responses during polyp bailout. Comparison between the two stressors

showed that certain signaling pathways are independent of the stressor and suggested that polyp bailout is a general response of corals to acute stress. Furthermore, immune system responses during polyp bailout indicate that microbe-associated partners of corals may lead to the polyp bailout response.

Keywords Polyp bailout · Transcriptome · Stress response · Signaling pathways

Introduction

Scleractinian corals are sessile modular invertebrates, currently facing severe environmental change with rising CO₂ levels and sea surface temperatures (Pandolfi and Kiessling 2014; Hughes et al. 2017). Corals are vulnerable to drastic environmental changes, due to their sessile life-style and the associated inability to move away during stress periods. Hence, corals have evolved various life-history traits concerning reproduction, growth, or competition, which aid survival and mitigate stressful conditions (Baird et al. 2009; Hidaka 2016).

One remarkable stress response, “polyp bailout” (Sammarco 1982), allows coral colonies to overcome disadvantages of their sessility (Jackson and Coates 1986; Hidaka 2016). Polyp bailout enables the coral to revise its sessile modular state (Hall and Hughes 1996) and return to mobile pelagic stages, potentially allowing the coral to escape unfavorable conditions. After degradation of the coenosarc individual polyps can detach themselves completely from the skeleton. Polyps retain their zooxanthellae, can reattach themselves to a surface and grow into a new colony (Sammarco 1982; Wecker et al. 2018). Studies in the corals *Seriatopora* and *Pocillopora* showed that the

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✉ Fabian Gösser
Fabian.Goesser@rub.de

¹ Department of Animal Ecology, Evolution and Biodiversity, University of Bochum, 44780 Bochum, Germany

² Department of Bioinformatics, University of Bochum, 44780 Bochum, Germany

detached polyps can survive and resettle, at least under laboratory conditions (Sammarco 1982; Shapiro et al. 2016). Polyp bailout seems to be a common stress response of various corals (e.g., Sammarco 1982; Serrano et al. 2018). The most studies, however, have been based on (incidental) laboratory observations. It has been reported that polyp bailout is inducible with multiple stressors including acidification, hypersalinity, and thermal stress (Kvitt et al. 2015; Shapiro et al. 2016; Fordyce et al. 2017). In the past decade, *Pocillopora* was a frequent choice to study polyp bailout and its accompanying regulatory pathways.

Polyp bailout was described in *Pocillopora* under reduced pH conditions (pH 7.2) leading to high levels of caspase activity, which were the most likely responsible for the apoptotic processes leading to the dissolution of the coenosarc (Kvitt et al. 2015). Wecker et al. (2018) conducted a transcriptomic stress study with *Pocillopora*. Concentrations higher than $30 \mu\text{g L}^{-1}$ of the insecticide chlordecone, led to the dissolution of the coenosarc followed by polyp detachment (Wecker et al. 2018). Overexpression of transcripts involved in apoptosis and in the degradation of cellular matrix proteins was found and supported the assumptions made by Kvitt et al. (2015). Based on the results of Kvitt et al. (2015), Shapiro et al. (2016), and Wecker et al. (2018), a recent study used hypersalinity to induce polyp bailout in *Pocillopora acuta* and added further information about the signaling pathways. Beyond upregulation of apoptosis and proteolysis, *P. acuta* showed significant activation of tumor necrosis factor and fibroblast growth factor, which probably led to the polyp bailout response (Chuang and Mitarai 2020).

The studies on Pocilloporidae used various artificial stressors (Wecker et al. 2018; Chuang and Mitarai 2020), most of which do not occur in natural systems. However, it is important to investigate polyp bailout under ecologically relevant and naturally occurring stress regimes to better understand the signaling pathways leading to this specialized stress response. In the present study, we used hypersalinity and heat to induce polyp bailout in *P. acuta*. Using transcriptomic data and a qPCR (quantitative polymerase chain reaction) assay, we aimed to identify the signaling pathways associated with polyp bailout. To identify the genetic mechanisms behind this stress response, we compared the signaling pathways induced by heat stress with those induced by hypersalinity. In addition, we aimed to find similarities in the gene expression induced by each stressor to provide a partial snapshot of the dynamic cellular machinery in action during polyp bailout.

Materials and methods

Coral collection and maintenance

Pocillopora acuta colonies were purchased (Korallenfarm Joe & Co, Witten, Germany) and kept in a 2000 L flow-through mesocosm (12 h day–night light cycle, 35‰ salinity, 25 °C). Ten percent of the water was exchanged weekly with artificial seawater (Red Sea Coral Pro Salt, Germany). Water chemistry was checked three times a week and adjusted with balling salts (Preis Aquaristik KG, Germany) or nutrient solutions (KNO_3 , K_2HPO_4). Colonies in the mesocosm were fed twice a week with freshly hatched *Artemia* sp. or Coral V Power (Preis Aquaristik KG, Germany).

Polyp bailout induction and tissue sampling

Prior to experiments, ten coral colonies of *P. acuta* were screened to perform bailout during temperature and hypersalinity stress. One colony was selected for the experiments, broken into 1.5 cm^2 fragments which then regenerated for 14 days. The first experiment induced polyp bailout using temperature stress. 6 L indoor-aquaria (three technical replicates per treatment) were adjusted to 25 °C (ambient conditions), 30 °C (elevated temperature), or 34 °C (heat stress). Each aquarium was illuminated with a 12 h day–night cycle and filled with artificial seawater at 35‰ salinity. Temperatures fluctuated no more than ± 0.5 °C per treatment per day. For each of the three technical replicates, nine fragments were placed in the ambient condition aquaria. After 24 h, all fragments were briefly lifted out of the water and at random three fragments were returned to the ambient aquaria and six were transferred to the elevated-temperature treatment. After a further 24 h, fragments were briefly lifted out of the water; the three ambient fragments were returned to their aquaria, while the six elevated-temperature fragments were randomly split between the elevated-temperature treatment and the heat-stress treatment. Fragments under heat stress were monitored and collected as soon as the coenosarc started dissolving (~ 16 h stress exposure), before being fixed in liquid nitrogen and stored at -80 °C (Fig. 1).

The second experiment induced polyp bailout via hypersalinity as described by Shapiro et al. (2016). Petri dishes (three replicates per treatment) were filled with 15 mL artificial seawater at 35‰ salinity. Each petri dish was illuminated with a 12 h day–night cycle and kept at 25 °C. Eighteen fragments were randomly distributed resulting in three fragments per petri dish. Three of the petri dishes were used as control treatments and covered with a lid to prevent evaporation. The remaining three petri

dishes were left uncovered to allow evaporation and, hence, reduce the water level. Due to evaporation, salinity increased over time (~ 16 h) from 35‰ up to 54‰ salinity. Fragments were closely monitored after salinity increase to 45‰ and collected directly after coenosarc dissolving (54‰), fixed in liquid nitrogen, and stored at -80°C . Detached polyps from both experiments were transferred to ambient conditions. Vitality and survival of polyps were monitored for two more days.

Additionally, fragments of the same colony were treated with five different stressors (Supplement Table S1) to obtain differently stressed coral fragments for transcriptome assembly. The sequences obtained from these stress libraries were later used to help assemble as complete transcriptome as possible, but were not used further for differential gene analysis.

RNA extraction, Illumina RNA sequencing, and transcriptome assembly

Coral fragments were crushed in 500 μL of TRIzol (Thermo Fisher Scientific, USA) and lysed according to the manufacturer's instructions. RNA was precipitated by adding 1 mL isopropanol absolute, purified using the RNeasy Mini Kit (QIAGEN GmbH, Germany) according to the manufacturer's instructions (including an additional DNase digestion step), and checked with a Qubit 3.0 fluorometer (Thermo Fisher Scientific, USA).

Samples per treatment were pooled (heat: three treatments $\times$ three biological replicates; hypersalinity: two treatments $\times$ two biological replicates; general stressors three pools of five stressors) resulting in 16 pooled samples. All pooled samples were used for library construction using the NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina® (New England Biolabs, USA) according to the manufacturer's protocol. Successful library construction was checked using a 5200 Fragment Analyzer System (Agilent Technologies, USA). Libraries were sent to Macrogen (South Korea) for 2×150 bp sequencing on an Illumina HiSeq 2500 sequencing platform. About 190 million demultiplexed read pairs (379,046,670 raw sequences) were received after sequencing, trimmed using Cutadapt v1.16.3 (Martin 2011) to remove adapters, low-quality sequences (Phred score < 30) and short reads (< 75 bp), and checked using FastQC (Andrews 2010), leaving a total of 87.5% read pairs (331,752,428 reads). The reads were assembled using Trinity v2.8.4 (Grabherr et al. 2011) and functionally annotated using the SwissProt eukaryotic protein database with BLASTX, using a threshold of $E = 10^{-5}$. The assembled transcripts were clustered for representative sequences using CD-HIT (Li and Godzik 2006) with a similarity threshold of 0.95. Transcriptome completeness was evaluated with BUSCO analysis (Simão et al. 2015) and the metazoa_odb10 dataset.

Fig. 1 Heat-stress-induced polyp bailout in *Pocillopora acuta*. After 24 h in 30°C , fragments were transferred to 34°C aquaria. Sampling points of the qPCR assay are indicated in gray, and number of sampled fragments in black. At each sampling point, equivalent numbers of fragments were taken from the control group (ambient, 25°C)

After 8–12 h, thinning of coenosarc could be observed (arrow). After 16 h, polyp bailout started. Polyps detached either as single polyps or patches. Samples of polyps were taken after another 8 h in the ambient conditions

Differential gene expression and gene ontology enrichment analyses

Trimmed reads were filtered for rRNA contamination using SortMeRNA (Kopylova et al. 2012) against the SILVA ribosomal RNA database (Quast et al. 2012). Remaining reads were mapped against the *Cladocypium goreau* genome (Chen et al. 2019) using HiSat2 (Kim et al. 2015) to exclude Symbiodiniaceae-derived hits. Reads with no match were mapped against our assembled and clustered transcriptome using HiSat2 and transcripts were quantified with featureCounts (Liao et al. 2014). Differential gene expression analysis was performed using DESeq2 (Love et al. 2014). Given that the two stress experiments differed in both experimental design and temporal execution, we analyzed differential gene expression in two independent models. The comparison of genes differentially expressed in both stress experiments helps us identify genes suspected to be involved in polyp bailout rather than a specific stress response of the treatments used. First, the counts of the heat-stress libraries were used against the respective elevated temperature and ambient libraries and a second time using the counts from the hypersalinity stress libraries against the corresponding ambient libraries. Outputs were filtered for transcripts with an absolute $\log_2$ fold change > 1 and an adjusted p value < 0.05 . Filtered outputs from both experiments were compared to identify genes involved in polyp bailout. Heatmaps were constructed with the “ComplexHeatmap” package (Gu et al. 2016) with RStudio (RStudio Team 2020). We compared the transcripts of ambient conditions and hypersalinity with the conditions of the elevated temperature and heat stress to identify those differentially expressed in both stress experiments. The sequences of the ambient conditions in the heat-stress experiments are not shown (Fig. 2) because they were almost identical to the sequences under elevated conditions (Supplementary Figure S2). Only transcripts that showed differential gene expression during both stress experiments (abs. $\log_2$ fold change > 1 , adjusted p value < 0.05) were selected, as these are likely to be involved in the polyp bailout response rather than in specific stress responses. The transcripts were annotated functionally with the SwissProt blast file that we created during the annotation of the transcriptome. For genes identified in multiple transcripts, the transcript with the highest absolute fold change was selected as it is likely to have the greatest biological significance and to obtain a list of unique gene identifiers for gene ontology enrichment analysis differentially expressed in both treatments. The list of unique gene identifiers was then used in the analysis of cellular processes and signaling pathways using DAVID bioinformatics resources v6.8 (Da Huang et al. 2009) for functional gene ontology (GO) enrichment analyses (Ashburner et al.

2000) against the genome backgrounds provided by DAVID using a modified Fisher’s exact test.

Real-time quantitative PCR analysis of temperature-induced polyp bailout induction

To increase temporal resolution of polyp bailout, we used real-time quantitative PCR (RT-qPCR). The temperature stress experimental setup was identical to that described previously. A second *P. acuta* genotype was used. Two technical replicates were used in each of the six experimental runs. Samples were collected after 24 h in the elevated-temperature treatment and after 8 h and 16 h in the heat-stress treatment. Control fragments were sampled at the same time points from the ambient conditions. In three of the six replicates, two additional time points were sampled: 4 h and 12 h after stress exposure. Detached vital polyps were collected from the heat-stress experiment, transferred to ambient conditions, and sampled after 8 h in ambient conditions (24 h after initiation of heat stress, $N = 3$). Samples were fixed in liquid nitrogen and RNA was extracted using the Monarch Total RNA Miniprep Kit (New England Biolabs, USA) according to the manufacturer’s instructions and checked for integrity and concentration by photometry (BioPhotometer, Eppendorf, Germany).

cDNA synthesis and RT-qPCR

For the RT-qPCR assay, we chose four genes and one reference gene (MMP25, NFKB1, TNFR6, CASP8, and TUBB2) representing three categories of interest for polyp bailout (Table 1) from Chuang and Mitarai (2020). We complemented the assay with seven genes (TRAF2, TNFRSF19, MMP12, DNJC3, BC2L, DDX58, and FGF10; Table 1) from our RNAseq data using Geneious V 11.1.3 (www.geneious.com). RNA of each sample was diluted to $10 \text{ ng } \mu\text{L}^{-1}$, and the RT-qPCR reaction was conducted with the Luna Universal One-step RT-qPCR Kit (New England Biolabs, USA). For each qPCR reaction, $5 \text{ } \mu\text{L}$ buffer ($2\times$), $0.4 \text{ } \mu\text{L}$ of each primer ($10 \text{ } \mu\text{M}$), $2.7 \text{ } \mu\text{L}$ RNase-free water, $0.5 \text{ } \mu\text{L}$ RT-Enzyme Mix, and $1 \text{ } \mu\text{L}$ of diluted RNA were used in a total volume of $10 \text{ } \mu\text{L}$. All qPCR reactions were performed on a LightCycler 96 System (Roche Molecular Systems, USA) with the following protocol: $55 \text{ } ^\circ\text{C}$ for 600 s; $95 \text{ } ^\circ\text{C}$ for 60 s; 45 cycles at $95 \text{ } ^\circ\text{C}$ for 10 s, and $60 \text{ } ^\circ\text{C}$ for 60 s followed by a melting step at $97 \text{ } ^\circ\text{C}$. For each gene in the qPCR assay, the mean cycle threshold (CT) value from two technical replicates of a given sample was taken to calculate the relative gene expression. qPCR primer efficiencies were calculated using LinRegPCR v. 2018.0 (Ruijter et al. 2009). The actual relative gene expression values between the reference gene

Fig. 2 Heatmaps constructed with Z-scores from the normalized counts of statistically significant genes ($p \leq 0.05$, abs. FC > 2) for hypersalinity (a), heat stress (b) and DEGs present in both treatments

(c). The Venn diagram shows the overlap (violet) between DEGs from hypersalinity-induced polyp bailout (blue) and heat-stress-induced polyp bailout (red)

and the genes of interest between ambient conditions (control) and treatments at given time points were calculated and tested using a pairwise fixed reallocation randomization test implemented in REST2009 (Pfaffl et al. 2002). $\log_2(\text{values} + 1)$ of relative gene expression were

plotted with the ggplot2 package (Wickham 2016) in RStudio (RStudio Team 2020). Further differential expression differences within a gene between the individual time points were calculated using in REST2009 (Pfaffl et al. 2002) (Supplement Table S2).

Table 1 Primers used in the qPCR assay

Genes of interest	Uniprot ID	Primer name	Primer sequence (5'–3')	Reference transcript	Fragment size (bp)	Efficiency	Gene category
FGF10	FGF10_HUMAN	FGF10_1123F ^b FGF10_1123R ^b	ACACGGATTGGGAAGAGCTG CTCCTGCCACATCTCCTTCC	TRINITY_DN2527_c0_g1_i5	145	0.92	ECM organization
MMP12	MMP12_HUMAN	MMP12_1481_3F ^b MMP12_1481_3R ^b	CTCAACCAAGATCCCAACGCCT GCACGTGTAAAGTGCATCG	TRINITY_DN222_c0_g1_i1	198	0.99	
MMP24	MMP24_RAT	MMP24_F1 ^a MMP24_R1 ^a	TCCAATGGGAAGAACGGCAG TGAGTAAAGATGCCATGAAGAG	20183_c0_g1_i1	218	0.96	
DDX58	DDX58_MOUSE	DDX58_217F ^b DDX58_217R ^b	CATCGAGGCGACACAGAACT CGGGTCCAAACAACAAGCAG	TRINITY_DN3863_c1_g1_i2	148	0.99	Inter-kingdom communication
NFKB1	NFKB1_CHICK	NFKB1_F1 ^a NFKB1_R1 ^a	CCTTTTGTACCAAGTGCAGTG TGTCATGAATCTGTTAGAGGC	27043_c0_g1_i2	190	1.00	Negative regulation of apoptosis
BCI2L1	B2CL1_MOUSE	B2CL1_1316F ^b B2CL1_1316R ^b	AAACTGGGTGCGCATAGTGG GAAACCATCCCATCCACCGT	TRINITY_DN3130_c0_g1_i6	172	1.00	
TRAF2	TRAF2_HUMAN	TRAF2_2101F ^b TRAF2_2101R ^b	GACGTGATGCACAGTCAGGA GGAGCATCGCGTCATACTGA	TRINITY_DN923_c0_g1_i7	174	1.00	
DNAJC3	DNAJC3_HUMAN	DNAJC3_172F ^b DNAJC3_172R ^b	AGATTGCTTGCCACTGGACA GCAAGGCAGCTTTTGAAACGA	TRINITY_DN6771_c0_g1_i6	139	1.00	
FAS	TNR6_PIG	TNR6_F1 ^a TNR6_R1 ^a	GCCAAACAACCTCAGGAGACAC GCAAAGCAATTACCGCAAAAC	4317_c14_g1_i1	211	0.98	Positive regulation of apoptosis
CASP8	CASP8_HUMAN	CASP8_F1 ^a CASP8_R1 ^a	AATGAACACTTCCAGGGAAAC GGCTAAGCAAGCAGCAAAAC	22697_c0_g1_i1	207	1.00	
TNFRSF19	TNFR19_HUMAN	TNFR19_1983F ^b TNFR19_1983R ^b	AGCAGGTCAAGGTACATGGC AAACATACGGCTCCCTTGGG	TRINITY_DN404_c1_g2_i4	170	0.99	
TUBB	TBB_PARLI	TUBB_F2 ^a TUBB_R2 ^a	GCAGTTACCGGTATGTTC TTTTCACCCCTCCTCTTCCTC	36257_c3_g1_i1	188	0.96	Reference gene
LIG3	DNLI3_HUMAN	DNLI3_316_1F ^b DNLI3_316_1R ^b	GAGGTTCTCAAATCCGCCCA GCTGCACAGGGAAGAGCTAA	TRINITY_DN5477_c0_g1_i11	216	1.00	

^aChuang and Mitarai (2020)^bThis study^cReference genes

Results

Polyp bailout induction

To compare differentially expressed genes and active signaling pathways leading to polyp bailout, we induced polyp bailout in *P. acuta* with hypersalinity and heat stress. During hypersaline stress, polyp retraction occurred at 42–46%. Significant thinning of the coenosarc was observed around 50%, and dissolution of the coenosarc began above this. Finally, at a salinity of 54‰ (16 h), partial dissolution of the coenosarc tissue and detachment of polyps were observed (Fig. 1). No physiological changes were observed in the heat-stress experiment at 24 h in 30 °C. After transfer to 34 °C tanks, thinning of the coenosarc was observed after 8–12 h. After 16–17 h, dissolution of the coenosarc and detachment of the tissue from the skeleton began. Additionally, for our qPCR assay, detached vital polyps were sampled 8 h after the occurrence of polyp bailout. For the control groups in the experiments with hypersalinity stress as well as heat stress, salinity changes during the experimental period were < 1‰ and temperature fluctuations were < 1 °C. In the control experiments, the tentacles were extended and no signs of polyp bailout or other stress responses like bleaching were observed.

RNA extraction, Illumina RNA sequencing, and transcriptome assembly

Filtering of low-quality reads and trimming of adapters left a total of 87.5% read pairs (331,752,428 reads). 714,514 transcripts were reconstructed (E90N50 = 1088nt) with 96.4% transcriptomic completeness; 1.8% fragmented; 1.8% missing; identified by the BUSCO analysis. Clustering of the transcript for representative sequences left 529,629 transcripts with 96% transcriptomic completeness (1.8% fragmented; 2.2% missing) identified by the BUSCO analysis. For the differential gene expression (DE) analysis, trimmed reads were filtered against the SILVA rRNA database (average 3.2% rRNA reads per sample) and mapped against the *Cladocopium goreau* genome (average 12.9% reads per sample) and were excluded.

Differential gene expression analyses

We found 6408 unique differentially expressed genes (DEG) in our hypersalinity stress experiment (Salinity 54‰ vs. Ambient 35‰, Supplement Figure S1) and 5043 DEG in our heat-stress experiment (Heat vs. Elevated and Ambient, Supplement Figure S1). The comparison of differentially expressed genes present in both experiments

showed an overlap of 2130 unique DEGs. In our hypersalinity experiment, we observed the formation of three different expression clusters (Fig. 2). Salinity Cluster 1 (2700 DEG) was upregulated, while the other two clusters were downregulated (Salinity Cluster 2: 1654 DEG; Salinity Cluster 3: 2054 DEG). For the heat-stress experiment, we found 5043 DEGs, which also clustered into three distinct profiles (Fig. 2). Heat Cluster 3 (842 DEG) was upregulated, while Heat Cluster 1 (2478 DEG) was downregulated. Heat Cluster 2 (1723 DEG) showed a large upregulation for one sample (Heat2, Fig. 2b) and only mild upregulation for the other two (Heat1, Heat3, Fig. 2b). To determine which genes are involved only in the polyp bailout response, we identified genes significantly differentially expressed in both experiments. 2130 DEG were identified in the analysis of both experiments and clustering of the transcripts led to the formation of four distinct clusters (Fig. 2c). Two clusters showed upregulation of genes (Combined Cluster 1: 548 DEG, Combined Cluster 2: 563 DEG), one showed downregulation (Combined Cluster 4: 628 DEG), while another showed opposing trends for the hypersalinity stress in comparison to the heat stress (Combined Cluster 3: 302 DEG). Almost exclusively all metallo-matrix proteases (MMP) are found in the two upregulated clusters (16 MMP in Combined Clusters 1 and 2; 2 MMP in Combined Cluster 3; Fig. 2c).

Gene ontology enrichment analyses

Gene ontology (GO) enrichment analysis was conducted to identify molecular mechanisms involved in polyp bailout (Supplement Table S3). Because we cannot rule out the possibility that enriched GO terms in our results are related to general stress responses of corals, we refer below to signaling pathways that have been previously discussed as involved or likely involved in polyp bailout, including regulation of apoptosis, proteolysis, and cell responses leading to survival (Kvitt et al. 2015; Wecker et al. 2018; Chuang and Mitarai 2020). Among upregulated DEGs in the hypersalinity stress (Salinity Cluster 1), we found GO categories already described for polyp bailout in this stressor (Chuang and Mitarai 2020), such as tumor necrosis factor (TNF)-mediated signaling pathway, fibroblast growth factor (FGF) receptor signaling pathway, extrinsic apoptotic signaling pathway, and proteolysis, which were significantly enriched ($p < 0.05$). Among downregulated DEGs (Salinity Clusters 2, 3), significant enrichment was identified in several GO categories related to cellular metabolism and the cell cycle. For the heat stress, upregulated clusters (Heat Clusters 2 and 3) showed similar GO terms as in the hypersalinity stress. Regulation of apoptotic processes, extrinsic apoptotic signaling pathway, and proteolysis were significantly enriched ($p = 0.05$). In the

Table 2 Selected functional enriched gene ontology categories among all differentially expressed genes (DEGs) present in both stress experiments (hypersalinity vs. heat)

Category	GO ID	Count	<i>p</i> value	FE
<i>Cellular functions</i>				
metabolic process	GO:0008152	383	8.2E−04	1.10
cell differentiation	GO:0030154	146	9.7E−04	1.27
<i>Response to stress/ROS</i>				
DNA repair	GO:0006281	23	1.1E−02	1.38
response to oxidative stress	GO:0006979	23	6.8E−03	1.85
response to reactive oxygen species	GO:0000302	14	1.6E−02	2.12
<i>Tissue degradation and remodeling</i>				
protein ubiquitination	GO:0016567	40	2.4E−03	1.65
proteolysis	GO:0006508	78	5.7E−04	1.46
regulation of extracellular matrix organization	GO:1903053	6	2.6E−03	6.14
tissue development	GO:0009888	72	1.6E−02	1.30
<i>Signaling pathways</i>				
I-kappaB kinase/NF-kappaB signaling	GO:0007249	14	6.3E−02	1.73
JNK cascade	GO:0007254	12	3.0E−02	2.08
regulation of MAPK cascade	GO:0043408	29	8.5E−02	1.36
<i>Apoptosis</i>				
apoptotic process	GO:0006915	65	1.1E−02	1.18
extrinsic apoptotic signaling pathway	GO:0097191	9	4.1E−02	1.27
regulation of tumor necrosis factor-mediated signaling pathway	GO:0010803	5	7.6E−02	3.11
tumor necrosis factor production	GO:0032640	7	9.9E−02	2.20
<i>Immune response</i>				
multi-organism process	GO:0051704	76	4.5E−02	1.03
symbiosis, encompassing mutualism through parasitism	GO:0044403	40	9.4E−02	1.27
pattern recognition receptor signaling pathway	GO:0002221	13	4.5E−03	2.60
toll-like receptor signaling pathway	GO:0002224	8	8.2E−02	2.13
MyD88-dependent toll-like receptor signaling pathway	GO:0002755	3	2.8E−02	2.89
immune system process	GO:0002376	68	9.2E−02	1.88
innate immune response	GO:0045087	23	8.3E−02	1.87
<i>Regeneration and wound healing</i>				
wound healing	GO:0042060	21	2.6E−02	1.23
Wnt-signaling pathway	GO:0016055	18	3.1E−02	1.22
fibroblast growth factor receptor signaling pathway	GO:0008543	7	9.9E−02	2.20
Ras protein signal transduction	GO:0007265	7	9.4E−01	0.69
epidermal growth factor receptor signaling pathway	GO:0007173	6	3.3E−02	1.59
regulation of epithelial cell proliferation	GO:0050678	15	9.1E−02	1.00

Shown are GO terms functionally enriched ($p < 0.1$) that are deemed potentially relevant in the polyp bailout response

GO ID, number of DEGs (Count), p values, and fold enrichment (FE) are given for each GO category

downregulated cluster (Heat Cluster 1) GO terms related to cellular and metabolic processes, cell differentiation, and DNA repair were found, as were terms related to TNF production and signaling, and regulation of apoptotic signaling pathway. The list of shared genes differentially expressed in both stress experiments led to a total of 227 annotation clusters in the GO term enrichment analysis. We focus on GO terms that are most likely to be associated with the polyp bailout response, but reserve the right to

address additional GO terms potentially involved in polyp bailout. Noteworthy clusters are related to the ECM organization and disassembly, cell differentiation, response to stress, apoptotic processes, regulation of I-kappaB kinase/NF-kappaB signaling, symbiosis encompassing mutualism through parasitism, MAPK cascade, fibroblast growth factor receptor signaling pathway, regulation of proteolysis, and protein ubiquitination (see Table 2). Genes concerning protein ubiquitination and proteolysis are found in

all four combined clusters, as are genes connected to the broad GO term apoptotic processes. GO terms connected to the extrinsic apoptotic signaling pathway and its regulation are found only in the first cluster (Combined Cluster 1), while we find genes belonging to the GO term MAPK cascade in all clusters. The GO term I-kappaB kinase/NF-kappaB signaling and JNK cascade are absent from Combined Cluster 4. Though GO terms for DNA repair and cellular response to stress are present in all clusters, the term “wound healing,” describing genes involved in repairing tissue damage, is found only in Combined Cluster 2. The GO term “response to oxidative stress” is found in Combined Clusters 1, 3, and 4. GO terms related to multi-organism processes are found in all four clusters, especially those involved in symbiotic/mutualistic/parasitic interactions (see Table 2).

Quantitative PCR assay

For the 11 genes of interests in the qPCR assay (Table 1), gene expression changes showed trends equivalent with those identified in the mRNA sequencing analysis (Fig. 3). MMP24 and MMP12, both involved in proteolysis, showed significant differential expression after 8 h at 34 °C. After 12 h, expression increased even further, with a 50-fold change in expression, and remained at this high level at the time of polyp bailout (16 h) and in detached polyps (24 h). FGF10, which plays a role in wound healing, showed a significant upregulation after 4 h, with further significant increase after 12 h. At 16 h, it decreased but was still significantly upregulated and remained on this level in the detached vital polyps. DDX58, which is part of the RIG-I-like receptor signaling pathway, had an immediate significant upregulation after 4 h and slowly dropped in expression throughout the experiment. For genes related to negative regulation of apoptosis, respectively, cell survival and inhibition of caspases, we saw an immediate significant upregulation after 4 h. DNJC3 is significantly upregulated after 4 and 8 h but is significantly downregulated in the detached polyps after polyp bailout. Other genes in this category, TRAF2, B2CL1, and NFKB1 showed similar expression patterns to each other, although we see a decrease in expression level in the detached polyps for B2CL1 and NFKB1, while TRAF2 stays at the level observed during polyp bailout. The FAS gene is already significantly upregulated at the starting point, while CASP8 and TNFRSF19 showed significant upregulation after 4 h. Expression levels peaked in all genes at 12 h before dropping to levels observed at the start of the experiment in the detached polyps (Fig. 3).

Discussion

Induction of polyp bailout

Heat-stress-induced polyp bailout has only been shown in the two corals *Cladocora caespitosa* (Kruzic 2007) and in *Pocillopora damicornis* (Fordyce et al. 2017). We induced the polyp bailout by rapidly increasing the temperature up to 34 °C in *P. acuta*. Salinity-stress-induced polyp bailout has been described in four pocilloporid corals: *S. hystrix*, *S. pistillata*, *P. acuta*, and *P. damicornis* (Shapiro et al. 2016; Chuang and Mitarai 2020). Like Shapiro et al. (2016), we used evaporation driven hypersalinity stress and observed polyp bailout at comparable salinity increase from ambient condition (approximately 15‰). Further, our results show that a single genotype of *P. acuta* is capable of polyp bailout under at least two different extreme stress scenarios, and multiple genotypes are capable of heat-induced bailout. This suggests that polyp bailout may be a ubiquitous stress response to a variety of extreme stressors, which is supported by previous studies with different stressors in scleractinian corals (Kvitt et al. 2015; Wecker et al. 2018; Serrano et al. 2018).

Transcriptomic data on heat- and hypersalinity-induced polyp bailout, consisted of 5043 and 6408 DEGs, respectively. DEG discrepancy between the stressors is caused by higher variation between libraries in our heat-stress experiments. While 99% of variance between samples could be explained by the treatment in our hypersalinity analysis, only 75% of variance was explained by treatment in the heat experiment (Supplement Figure S1). Fragments were monitored continuously during the experiment and no visual signs of a bleaching reaction occurred. An explanation is that onset of stress response in one replicate was not synchronized with the other replicates due to minor temperature fluctuations. As results from our hypersalinity stress are very similar to the recently published study from Chuang and Mitarai (2020), we focus on the results and the signaling pathways of heat stress and the DEGs present in both stressors resulting in a list of 2130 DEGs (See Fig. 2).

General stress response

During heat stress as well as in our hypersalinity experiment, we found DEGs that are associated with coral responses to high-temperature regimes and general stress responses. Heat shock proteins (HSPs, e.g., HSP90, HSP40, HSP16), GFP, and GFP-like non-fluorescent protein and major oxidative stress proteins, are found among the significant DEGs. Heat stress is commonly associated with changes in the expression of HSPs, and higher expression levels can play a role in coral thermal tolerance

Fig. 3 Log₂(fold change+1) of expression $\pm$ SE of the genes of interest against time. The genes fit into four different biological categories: extracellular matrix (ECM) organization, inter-kingdom communication, negative regulation of apoptosis, and positive regulation of apoptosis. Black lines show the reference gene

expression. Differential gene expression to reference genes at a given time point was calculated using a pairwise fixed reallocation randomization test implemented in REST2009 software. Significant differences ($p < 0.05$) are indicated

(Barshis et al. 2013; Louis et al. 2017). A general down-regulation of housekeeping genes was observed, which could help minimize energy expenditure, allowing for efficient responses to stressful conditions, as housekeeping functions are generally thought to be very costly (Vidal-Dupiol et al. 2014). Differential downregulation of GFP and GFP-like non-fluorescent proteins is observed, as has been described previously under heat stress (Rodriguez-Lanetty et al. 2009). These genes can play a role in the oxidative stress response by acting as scavengers of reactive oxygen species (ROS) and may play an important role in maintaining cellular redox balance (Palmer et al. 2009). An intense increase in ROS can overwhelm defenses and trigger apoptosis and cell death in the coral host (Weis 2008). Hence, enrichment of the GO term for apoptotic

processes is not surprising in our heat-stress data, but it is also present during our hypersalinity experiment, leading to the presence of apoptosis-related genes and signaling pathways in our combined dataset. This is consistent with results from a hypersalinity experiment investigating signaling pathways during polyp bailout (Chuang and Mitarai 2020).

Underlying pathways of polyp bailout

Apoptotic signaling pathways

Metazoan apoptosis appears to be unique through its mediation by the TNF receptor family, activated by certain TNF ligands (Quistad and Traylor-Knowles 2016). The GO

term for the TNF signaling pathway, which has also been reported in other studies under heat stress (Vidal-Dupiol et al. 2014; Seneca and Palumbi 2015), is enriched in our heat-stress data and in our hypersalinity data, as already shown by Chuang & Mitarai (2020). TNF signaling can initiate a caspase cascade through activation of Caspase 8 and the downstream Caspase 3 (both present in our data), which has been proposed as the main reason for coenosarc degradation during polyp bailout (Wecker et al. 2018; Chuang and Mitarai 2020). We found no differential expression of TNFs, although we observed significant enrichment of the TNF signaling pathway and upregulation of the FAS receptor gene in our qPCR assay, which is similar to previous results (Chuang and Mitarai 2020). Genes responsible for the accumulation of the GO term “TNF signaling pathway” in our dataset perform tasks in the extrinsic apoptotic signaling pathway but can also be activated TNF-independently. In particular, several TNF-associated factors (TRAFs), which are the molecular link between various signaling pathways and the tumor necrosis factor receptors (Hauer et al. 2005), but are also involved in the regulation of the innate immune system (Barshis et al. 2013), come up in our gene list (for example, TRAF 1,2,3,6; Fig. 4).

Immunity and the holobiont

Stress or detection of injury trigger immune responses and can affect holobiont fitness (Palmer 2018). In our dataset enrichment of GO terms like “innate immune response” and “Toll-like receptor signaling pathway” suggest involvement of the innate immune system during polyp bailout and could also explain enrichment of the GO terms “MAPK cascade” and “JNK cascade”. Generally, Toll-like and RIG-I-like receptors are part of the innate immunity and can detect “microbe-associated molecular patterns” (MAMPs) and “danger-associated molecular patterns” (DAMPs) (Palmer 2018). Downstream signaling of these receptors, can also trigger caspase-related apoptotic processes (Park et al. 2007; Newton and Dixit 2012), but also regulate cell survival via the NFkB signaling pathway (Table 1, Fig. 4). Similar patterns have been described during coral stress reactions (van de Water et al. 2015; Williams et al. 2018) and polyp bailout (Wecker et al. 2018; Chuang and Mitarai 2020). The initial trigger and thresholds leading to polyp bailout mediated via MAMP/DAMP receptors might be still unknown, but it has been proven that changes in abiotic, or biotic factors can alter the composition of the holobiont, leading to changes in chemical signals delivered by microbes to the coral (Webster et al. 2013; Overstreet and Lotz 2016). Heat stress in combination with the coral pathogen *Vibrio coralliilyticus* has been proven to cause tissue lysis (Ben-

Haim et al. 2003; Vidal-Dupiol et al. 2011, 2014), similar to the dissolution of the coenosarc during bailout, and to actually triggering polyp bailout (Gavish et al. 2018). In our qPCR, DDX58, a detector of virus-related MAMPs, was upregulated shortly after the onset of extreme stress. Involvement of a microbe-associated trigger of polyp bailout has already been proposed (Chuang and Mitarai 2020) and may explain why we have previously found polyp bailout preferentially in some specific coral species, such as pocilloporids. The “damage threshold hypothesis” (Palmer 2018) suggests that species that preferentially invest in growth and/or reproduction rather than immune system usually live closer to their damage threshold. Therefore, the damage threshold is reached earlier than in species that are considered more resistant. Thus, an immune response occurs earlier in a more susceptible holobiont, which can lead to dysbiosis (Palmer 2018).

Mediation of anti-apoptotic signaling.

Although it has been proposed that the anti-apoptotic response is induced by delayed TNF signaling after polyp bailout (Wecker et al. 2018), our results suggest that the tissue specificity of apoptosis during polyp bailout is due to spatial variation in anti-apoptotic signaling. Caspase activity is inhibited in coral polyps and signals are triggered that lead to their subsequent survival, results also described in hypersaline stress by Chuang and Mitarai (2020). Bcl-2 and related genes may play a role in this anti-apoptotic cell signaling (Dunn et al. 2006; Moya et al. 2015) as upregulation of Bcl-2 and Bcl-2-l1 in our transcriptomic data and our qPCR assay suggests. In addition to Bcl-2 and related genes, TRAFs, can also be active in downregulation of apoptotic signaling and suppression of caspase-associated apoptosis via activation of the NFkB pathway (Karl et al. 2014). NFkB1, a transcription factor that mediates cell survival and proliferation, and anti-apoptotic factors prevent inflammatory responses from causing more tissue damage than the actual stress signal (Oeckinghaus and Ghosh 2009). A similar expression pattern for TRAF2, Bcl2-l1, and NFkB1 as for Caspase 8 can be observed, indicating a simultaneous activation of pro- and anti-apoptotic signals in coral tissue. However, the expression patterns observed for DNJC3 (Fig. 3), which can attenuate endoplasmic reticulum (ER) stress but lead to apoptosis when overloaded in cnidarians (Oakley et al. 2017) leave ambiguities. It could be part of the early activation of anti-apoptotic signaling during polyp bailout or an early response to general stress. Elucidation of the involvement of all components of anti-apoptotic/cell survival signaling and their functional role during polyp bailout requires further investigation, as our analysis is only a snapshot of the signaling pathways.

Fig. 4 Potential pathways of the innate immune system leading to apoptosis and cell survival. MAMP/DAMP signals are recognized by receptors, exemplarily Toll-like receptors (TLR), and RIG-I-like receptor (gene: DDX58) and subsequently activate downstream signaling. Genes present under both stress regimes are shown in green; genes present in at least one stressor are shown in blue, and

genes not present in our data are shown in white. Activation of the MAPK and JNK cascade, as well as NFkB signaling leads to production of different transcription factors and cytokines in the nucleus that lead to various responses, such as inflammation, immune regulation, survival, and proliferation

Wound healing, regeneration, and ECM organization

ROS signaling and activation of the innate immune system seem to be involved in stress responses and are also likely involved in wound healing and regeneration (Wenger et al. 2014). ROS are able to modulate cell migration through activation of matrix metalloproteinases (MMPs), and proliferation through epidermal growth factor receptor (EGFR) signaling (Cordeiro and Jacinto 2013; Wenger et al. 2014). Genes such as transforming growth factor β (TGF β), fibroblast growth factor (FGF), epidermal growth factor (EGF), and associated receptors are differentially expressed. Binding to receptors leads to downstream

activation of signaling pathways such as RHO-GTPase-dependent pathways and Jun-N-terminal kinase (JNK), which are enriched in our GO term analysis and finally lead to gene expression patterns characteristic of wound healing (Cordeiro and Jacinto 2013). Sustained release of ROS can lead to Wnt-release in dying cells, activating β -catenin signaling that leads to proliferation of neighboring cells (Chera et al. 2009). Expression of Wnt and FGF ligands, appear to be integral components of wound healing and regeneration in Cnidaria (Chera et al. 2009; Luz et al. 2021). In our GO term enrichment analysis, we found the Wnt-signaling pathway as well as wound healing significantly enriched; furthermore, β -catenin is differentially

expressed under both stressors suggesting the involvement of these components in polyp regeneration after coenosarc dissolution. Accumulation of MMP and fibroblast growth factors (FGF) and receptor (FGFR) homologues, which play a role in regeneration and wound healing, have been described as involved in the detachment of polyps during polyp bailout (Chuang and Mitarai 2020). FGF signals could be transduced through the Ras-signaling pathway to activate MMPs that initiate subsequent ECM degradation and detachment of the polyp from the calcareous skeleton. Enrichment of GO terms as “FGF receptor signaling pathway,” “Ras protein signal transduction,” “Proteolysis” accompanied by several MMP-encoding genes confirms the results of Chuang and Mitarai (2020).

Survival of detached polyps

Activation of specific spatio-temporal apoptosis and anti-apoptosis signaling, as well as regeneration and detachment of polyps, can result in a multitude of vital polyps from polyp bailout. Polyps stemming from our heat-stress experiment in our qPCR assay were tested for differential expression of “genes of interest.” DDx58, which is responsible for the recognition of PAMPs, underwent a normalization of expression to almost the same level as at the beginning of our experiment. Thus, transferring the polyps into ambient conditions also seems to contribute to the rapid stabilization of the holobiont and decreasing pathogen-associated signals. Genes that we have linked to “positive regulation of apoptosis” have also been down-regulated indicating that no further tissue degradation appears to occur, which is in line with previous studies that reported a normalization of gene expression in the detached polyps (Chuang et al. 2021). Genes involved in downregulation of apoptotic signaling, are still significantly upregulated. The reason could be, their involvement in cell proliferation, or the downregulation of these genes occur at the post-translational level, as has already been suggested for the MMPs during polyp bailout, which also still have high expression levels comparable to Chuang and Mitarai (2020). Another explanation of the high expression of MMPs may be processes such as wound healing and restructuring of the polyp tissue. Shortly after leaving the calcareous skeleton, polyps appear to become spherical in shape (Fig. 1) (Shapiro et al. 2016; Chuang et al. 2021), suggesting partial restructuring of the ECM and cell organization.

Polyp bailout vs. coral bleaching: A question of holobiont immunity

Typically, heat stress is associated with a drastic stress response in corals, coral bleaching. Genes and signaling

pathways, such as “response to ROS,” that are differentially expressed in our dataset are involved in a variety of coral stress responses, but have also been described in coral bleaching (Weis 2008; Li et al. 2021; Rädcker et al. 2021). Further, involvement of apoptotic processes and activation of TNF signaling have been linked with the establishment and loss of symbiosis during coral bleaching (Weis 2008). By analyzing the heat-stress data, we found genes that are differentially expressed in the context of glutamate biosynthesis, such as glutamine synthetase (GS) and glutamate dehydrogenase (GDH). Destabilization of the underlying symbiotic nutrient cycle appears to contribute either directly or indirectly to dysbiosis between corals and Symbiodiniaceae (Rädcker et al. 2021). Although coral bleaching differs significantly in symptoms and consequences from the polyp bailout response, we hypothesize that both responses occur simultaneously, and the outcome is strongly influenced by the duration and intensity of the stressors. We also looked for differential expression in the algal partner of the holobiont, but only found downregulation of genes belonging to homeostasis functions and cell cycle (Supplement Table S4). Stress intensity may have been too high and the duration too short to trigger a Symbiodiniaceae response, as previously shown for corals under acute heat stress (Li et al. 2021). The involvement of the immune system in both stress responses thereby highlights once more that stress responses and tolerance of corals is part of holobiont immunity (Weis 2008; Palmer 2018).

Applications of polyp bailout

Polyp bailout could be a promising approach to generate micropropagates as a base for highly replicable experiments. Advantages include small space, temporal resolution, high reproducibility, rapid production of hundreds of genetically identical replicates, and preservation of coral tissue integrity including the holobiont (Shapiro et al. 2016). The identification of signaling pathways during polyp bailout provides the basis for developing alternative methods to induce polyp bailout. Stimulation of ECM secretion or delivery of appropriate ECM components could promote resettlement of detached polyps. Previous studies have shown that fibronectin- and laminin-like peptides promote tissue attachment in corals (Domart-Coulon et al. 2004). Also, the involvement of the immune system, the use of antibiotics, probiotics, or viruses may be considered in further research. Future research should go back to natural reef systems and investigate the natural occurrence and ecological consequences and potential of polyp bailout.

Polyp bailout could be a useful tool to revolutionize coral experiments, serve as a basis for assisted evolution, and offer application in reef restoration.

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Data accessibility Data supporting the findings of this study are available on the NCBI database with the following Bioproject number: PRJNA728564.

Declarations

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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