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Signaling pathways in the coral polyp bail-out response

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Abstract Polyp bail-out is a stress response exhibited by some pocilloporid corals, with mechanisms and consequences distinct from those of bleaching. Although induction of polyp bail-out has been demonstrated in the laboratory, molecular mechanisms underlying this response have rarely been discussed. We conducted genetic analyses of *Pocillopora acuta* during initiation of hyperosmosis-induced polyp bail-out, using both transcriptomic and qPCR techniques. Beyond upregulation of apoptosis and proteolysis, corals showed significant activation of tumor necrosis factor and fibroblast growth factor (FGF) signaling pathways during induction of polyp bail-out. In our qPCR analysis, a common upregulation profile, peaking at 43.0‰ salinity, was found in the FAS and CASP8 genes, whereas a different profile, showing significant upregulation up to 45.0‰, was displayed by matrix metalloproteinases and genes in the FGF signaling pathway. These results suggest parallel involvement of an extrinsic apoptotic signaling pathway and FGF-mediated extracellular matrix degradation in polyp bail-out. Furthermore, in the XIAP, JNK, and NFkB1 genes, we detected a third expression profile showing linear upregulation that becomes maximal at the endpoint salinity level of the experiment (46.0‰), indicating activation of anti-apoptotic and cell survival signals

during polyp bail-out. Our results provide new insights into signaling pathways responsible for polyp bail-out and suggest the feasibility of inducing bail-out by specifically triggering these pathways without exerting lethal stresses on the corals, which in turn will facilitate acquisition of viable polyps for possible use in coral reef restoration.

Keywords Polyp bail-out · Apoptosis · ECM degradation · Signaling pathway · Transcriptome

Introduction

Corals in the family Pocilloporidae (Cnidaria: Anthozoa: Scleractinia) are among the major tropical reef-building corals in the Indo-Pacific Ocean (Pinzon and LaJeunesse 2011). As pocilloporid corals generally inhabit shallow water, which renders them susceptible to thermal and osmotic fluctuations (Schmidt-Roach et al. 2014; Poquita-Du et al. 2019), studies of stress responses in pocilloporids are of great importance to understand anthropogenic impacts on coral reef ecosystems. Coral bleaching, which is the systematic dissociation of endosymbiotic dinoflagellates from their coral hosts, is a general response of stony corals to a vast number of environmental stresses (Lesser et al. 1990; Jones and Hoegh-Guldberg 1999; Jones 2005; Ainsworth et al. 2008). As most stony corals rely on photosynthesis from symbiotic algae to meet their carbon requirements, coral bleaching is responsible for the high mortality rate among tropical corals (Hoegh-Guldberg 1999; Carpenter et al. 2008).

In recent decades, a novel stress response called “polyp bail-out” has been increasingly reported in pocilloporid corals (Sammarco 1982; Domart-Coulon et al. 2004; Kvitt et al. 2015; Shapiro et al. 2016; Fordyce et al. 2017;

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Wecker et al. 2018). Unlike coral bleaching, polyp bail-out is characterized by dissociation of coral colonies via coenosarc degradation and detachment of zooxanthellate polyps from the calcareous skeletons (Sammarco 1982). In natural environments, polyp bail-out has been reported in the Great Barrier Reef and reefs along the Pacific coast of Costa Rica, although causes are uncertain (Sammarco 1982; Wild et al. 2014). Under laboratory conditions, induction of polyp bail-out has been demonstrated with different treatments, including calcium deprivation, acidification, thermal stress, hyperosmosis, starvation, and insecticides (Domart-Coulon et al. 2004; Kvitt et al. 2015; Shapiro et al. 2016; Fordyce et al. 2017; Serrano et al. 2018; Wecker et al. 2018). As detached polyps can be maintained in the laboratory for weeks to months (Capel et al. 2014; Shapiro et al. 2016; Serrano et al. 2018), these polyps offer subject material for in situ studies of coral cellular biology and of symbiotic relationships between coral animals and symbiotic algae. Moreover, since detached polyps are able to resettle and resume skeletogenesis after stress abates, polyp bail-out can be considered an asexual reproductive method in stony corals and may provide an alternative approach to mass production of coral colonies for reef restoration (Sammarco 1982; Shapiro et al. 2016; Fordyce et al. 2017).

However, documented success of polyp survival and resettlement after bail-out varies greatly among studies (Sammarco 1982; Shapiro et al. 2016; Fordyce et al. 2017). These variations may be attributable to treatments used in different studies and to the quality of resettling environments, as well as the health of the corals in question (Shapiro et al. 2016). Understanding molecular mechanisms responsible for polyp bail-out is the first step in determining how different stimuli affect the process and consequences of this stress response. Identifying core signaling pathways leading to the response will also help to develop methods to induce the response with minimal impact upon coral health, which in turn will facilitate survival and resettlement of detached polyps. Recently, genomes and transcriptomes of some pocilloporid corals have been published (Traylor-Knowles et al. 2011; Voolstra et al. 2017; Cunning et al. 2018), enabling a more thorough understanding of this response from a molecular perspective. A recent transcriptomic study of *Pocillopora damicornis* during polyp bail-out demonstrated overexpression of many caspase-encoding genes in concert with coenosarc degradation (Wecker et al. 2018), supporting the hypothetical link between polyp bail-out and tissue-specific apoptosis revealed previously in a protein activity assay (Kvitt et al. 2015). In addition, Wecker et al. (2018) proposed that proteolytic enzymes, such as cathepsins, trigger degradation of the extracellular matrix (ECM) between coral polyps and the calcareous skeletons, resulting in

detachment of polyps. However, our understanding of molecular mechanisms in polyp bail-out, particularly signaling pathways activating apoptotic and proteolytic responses, is still in its infancy.

In the present study, we employed hyperosmotic stress to induce bail-out in *P. acuta*, a species closely related to *P. damicornis*, according to recent phylogenetic classifications (Schmidt-Roach et al. 2014; Johnston et al. 2017). Based on both transcriptomic and qPCR techniques, we sought to identify the signaling pathways associated with polyp bail-out.

Materials and methods

Coral collection and maintenance

During 2018–2019, we purchased nine colonies of *Pocillopora acuta* from the Onna Village Fisheries Cooperative in Okinawa, Japan. Coral colonies were transferred to the OIST Marine Science Section at Seragaki, where they were kept for over 6 months in a 3000-L outdoor tank supplied with flowing, sand-filtered natural seawater, before the commencement of polyp bail-out experiments.

Polyp bail-out induction and RNA sampling for transcriptomic analysis (Experiment I)

In order to examine molecular mechanisms underlying polyp bail-out, we induced the bail-out response in *P. acuta* using a hyperosmotic treatment modified from Shapiro et al. (2016). One day prior to the experiment, eight coral fragments (0.5–1 cm in length) from a mother colony were clipped off and randomly placed in two 5-L indoor experimental tanks (four in the treatment group and four in the control group). For both experimental tanks, light intensity was provided at $300 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (measured with an Apogee MQ-210 underwater quantum meter) with a 12-h day–night cycle. Artificial seawater (Kaisuimaren, Japan) at 35‰ salinity was prepared in both tanks, and water temperature was allowed to fluctuate daily in the range of 24–26 °C. Evaporation-driven hyperosmotic treatment, as reported in Shapiro et al. (2016), not only increases seawater salinity, but concentrates coral metabolic waste and microorganisms in seawater, which presumably constitute another stress on corals. Therefore, in the present study, we induced hyperosmotic stress by addition of hypersaline seawater. For the treatment group, high-salinity artificial seawater (48‰) was pumped into the experimental tank at a constant rate of 4 mL min^{-1} using a peristaltic pump, while for the control group, ambient salinity (35‰) was maintained. At 0 h, 12 h, and 24 h of the experiment, seawater salinity and temperature were

monitored with a ProfiLux 4 aquarium controller (GHL, Germany) and coral morphological changes were recorded using a stereo microscope (Leica, Germany). To examine genetic responses during initiation of polyp bail-out, one coral fragment was sampled at each of the time points from both treatment (samples labeled as salinity levels at the sampling points: T35.0, T43.0, and T46.0) and control groups (C35.0, C35.4, and C35.8) for subsequent Illumina RNA sequencing (Fig. 1). Since partial polyp detachment (an indicator of onset of polyp bail-out) was observed in the treatment group at 46.0‰ salinity (24 h) and bailed-out polyps could be easily removed from the skeleton by gently shaking the coral fragment in water, we collected both undetached coral tissues (whole fragment including undetached polyps, remaining coenosarc, and underlying skeleton; T46.0-U) and detached polyps (10–15 completely detached polyps, collected with a large-bore pipette; T46.0-D) separately. Coral samples were immediately preserved

in 1 mL TRIZOL reagent (Thermo Fisher Scientific, USA) after collection and were stored at -20°C until RNA extraction. To avoid artifacts from microscopic observation, a spare coral fragment in both treatment and control groups was used for morphological observation throughout the experiment and was not included in subsequent genetic analysis. From the spare coral fragment in the treatment group, we transferred detached polyps to the control tank immediately after their detachment. These polyps were later examined for viability via a stereo microscope (Leica, Germany). The same experiment was repeated three times on different *P. acuta* colonies, with at least 1 month between experiments ($N = 3$).

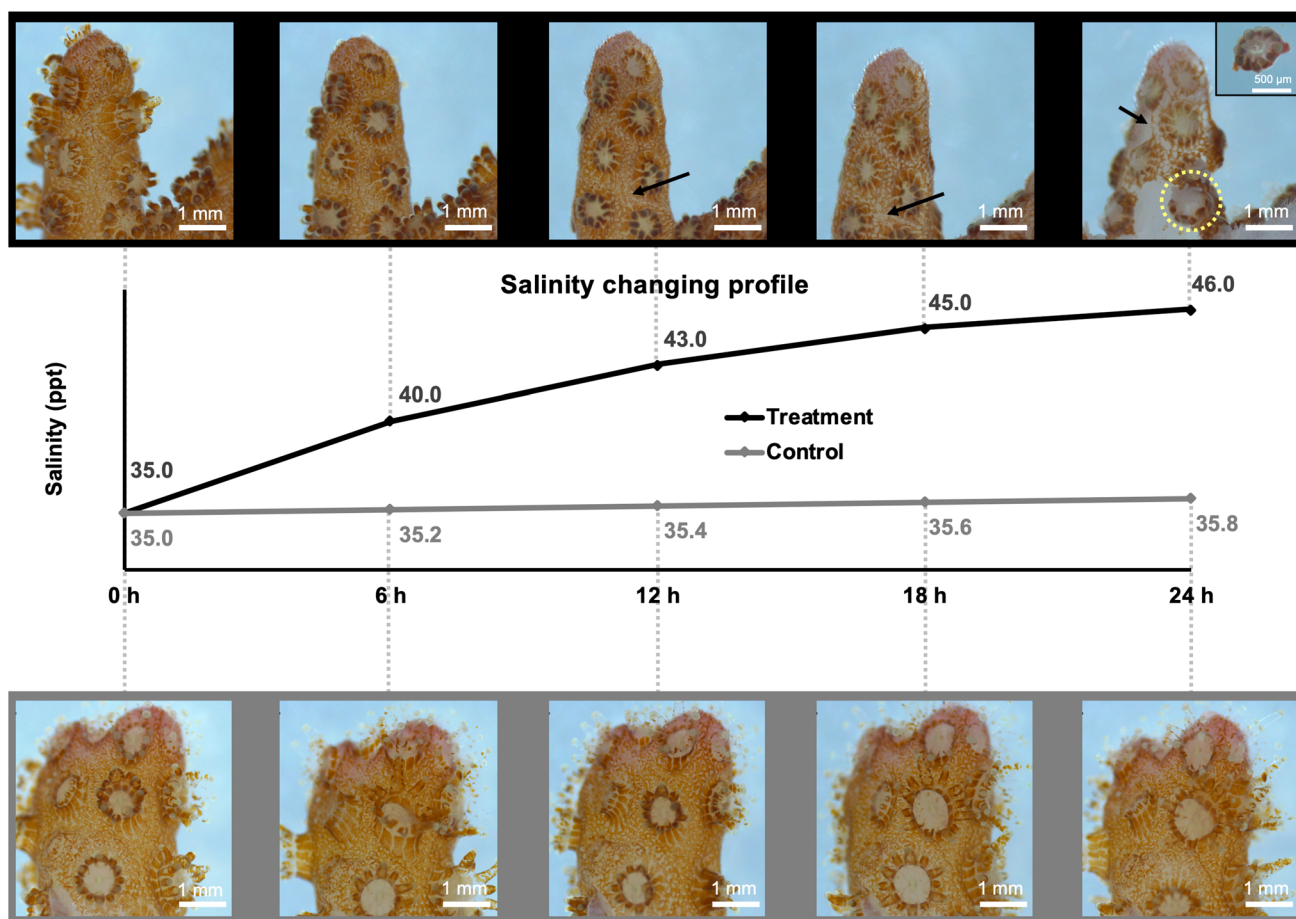


Fig. 1 Polyp bail-out in *Pocillopora acuta* under hyperosmotic stress. Seawater salinity changes in treatment and control groups during the experiment are presented in the central plot with black and gray lines, respectively. Coenosarc thinning/degradation (arrow) commenced in the treatment group when salinity reached 43.0‰ (12 h). Polyp detachment (yellow circle) was observed in the

treatment group at 46.0‰ salinity (24 h), yielding solitary, morphologically intact polyps (upper right box of treatment group at 46.0‰). Coral morphological changes were recorded on a spare coral fragment in both treatment and control groups at each sampling point using a stereo microscope

RNA extraction, Illumina RNA sequencing, and transcriptome assembly

For all tissue samples collected in this study, soft tissues were removed from the skeleton (if applicable) and homogenized in the TRIzol reagent using a beadblaster tissue homogenizer (2500 rpm for 2 min) before RNA extraction. Total RNA was extracted following the TRIzol RNA extraction protocol (Chomczynski 1993), and RNA quality was checked with a Bioanalyzer 2100 (Agilent, Japan). For Experiment I, RNA samples with RNA integrity number (RIN) ≥ 7 were sent to the DNA Sequencing Section at the Okinawa Institute of Science and Technology (OIST) for library construction (strand-specific and polyA-RNA purified; number of libraries = 7 experimental conditions \times 3 biological replicates) and Illumina HiSeq 4000 150 \times 150-bp paired-end RNA sequencing. Raw reads from Illumina RNA sequencing were trimmed with Trimmomatic v0.36 (Bolger et al. 2014) to remove adapters and low-quality sequences and were checked for quality using FastQC (Andrews 2010). To remove RNA reads from *Symbiodinium* and other coral-associated microbiomes, we first mapped trimmed RNA reads (\sim 877 million read pairs from all sequenced libraries) to a reference *Pocillopora damicornis* genome assembly (GenBank assembly accession: GCA_003704095.1) (Cunning et al. 2018) using Tophat v2.1.1 (Kim et al. 2013). Successfully aligned RNA reads (\sim 168 million read pairs; successful alignment rate: 19.1%) were then subjected to de novo assembly using Trinity v2.8.4 (Grabherr et al. 2011). RNA read quantification was conducted using RSEM v1.3.2 (Li and Dewey 2011), and reconstructed transcripts shorter than 200 nt or with low coverage (< 5 transcripts per million) were discarded. BUSCO analysis was performed using the metazoa_odb9 dataset to examine completeness of the transcriptome assembly (Simão et al. 2015).

Functional annotation, differential gene expression, and gene ontology enrichment analyses

We performed functional annotation of the transcriptome assembly by searching the SwissProt eukaryotic protein database (downloaded on July 3, 2019) with BLASTX, using a threshold of $E = 10^{-5}$. Principle component analysis (PCA) and a preliminary differential gene expression (DE) analysis of annotated transcripts showed that RNA-Seq libraries could be clearly classified based on salinity levels, despite a certain level of variation between biological replicates (Fig. 2). We therefore clustered RNA-Seq libraries into three groups, labeled with corresponding salinity levels (S35: T35.0, C35.0, C35.4, C35.8; S43: T43.0; S46: T46.0-U, T46.0-D). A secondary DE analysis was then conducted on annotated transcripts for pairwise

comparisons between the three groups (expressed as S35/S43, S35/S46, and S35/S43), designed to cancel out individual variation and to identify transcriptomic responses induced specifically by hyperosmotic treatment. Both preliminary and secondary DE analyses were performed using edgeR (Robinson et al. 2010) with the criteria of $>$ twofold absolute change and > 5 CPM (counts per million) for at least 3 libraries in a given pairwise comparison. To identify cellular processes and signaling pathways responsible for polyp bail-out, gene ontology (GO) enrichment analysis was performed for differentially expressed genes (DEG) using DAVID bioinformatics resources v6.8 (Huang et al. 2008, 2009).

Polyp bail-out induction for real-time quantitative PCR analysis (Experiments II and III)

To validate polyp bail-out and genetic responses induced by hyperosmosis, in 2019–2020 we repeated the polyp bail-out experiment under: (1) the same culturing conditions as Experiment I (artificial seawater at 35‰; 300 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$; Experiment II) and (2) different culturing conditions (filtered seawater at \sim 35‰; 150 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$; Experiment III). In each experiment, 36 fragments from three *P. acuta* colonies ($N = 3$; 12 fragments/colony) were randomly placed in two 5-L tanks 1 day prior to the experiment (6 in treatment group and 6 in control group for each colony). For better temporal resolution of genetic analysis, one fragment/colony was sampled from both treatment and control groups every 6 h during the experiment (Fig. 1), at which salinity levels (treatment group/control group) were 35.0‰/35.0‰ (0 h), 40.0‰/35.2‰ (6 h), 43.0‰/35.4‰ (12 h), 45.0‰/35.6‰ (18 h), and 46.0‰/35.8‰ (24 h). As in Experiment I, seawater salinity, temperature, and coral morphological changes (in the spare fragments) were recorded at each sampling point. Since the transcriptomes of undetached coral tissues (T46.0-U) and detached polyps (T46.0-D) collected in Experiment I showed no obvious differences in our preliminary analyses (Fig. 2 and Table S1), in Experiments II and III we collected undetached coral tissues and detached polyps together as one sample (treatment group; 46.0‰). Collected coral samples were preserved in TRIzol reagent as in Experiment I and were subsequently subjected to real-time quantitative PCR (qPCR) analysis.

cDNA synthesis and qPCR assay

For samples from Experiments II and III ($N = 3$ biological replicates \times 5 sampling points \times 2 experimental conditions (treatment vs. control) for each experiment), total RNA was extracted as described above and cDNA synthesis was conducted using the SuperScript IV VILO

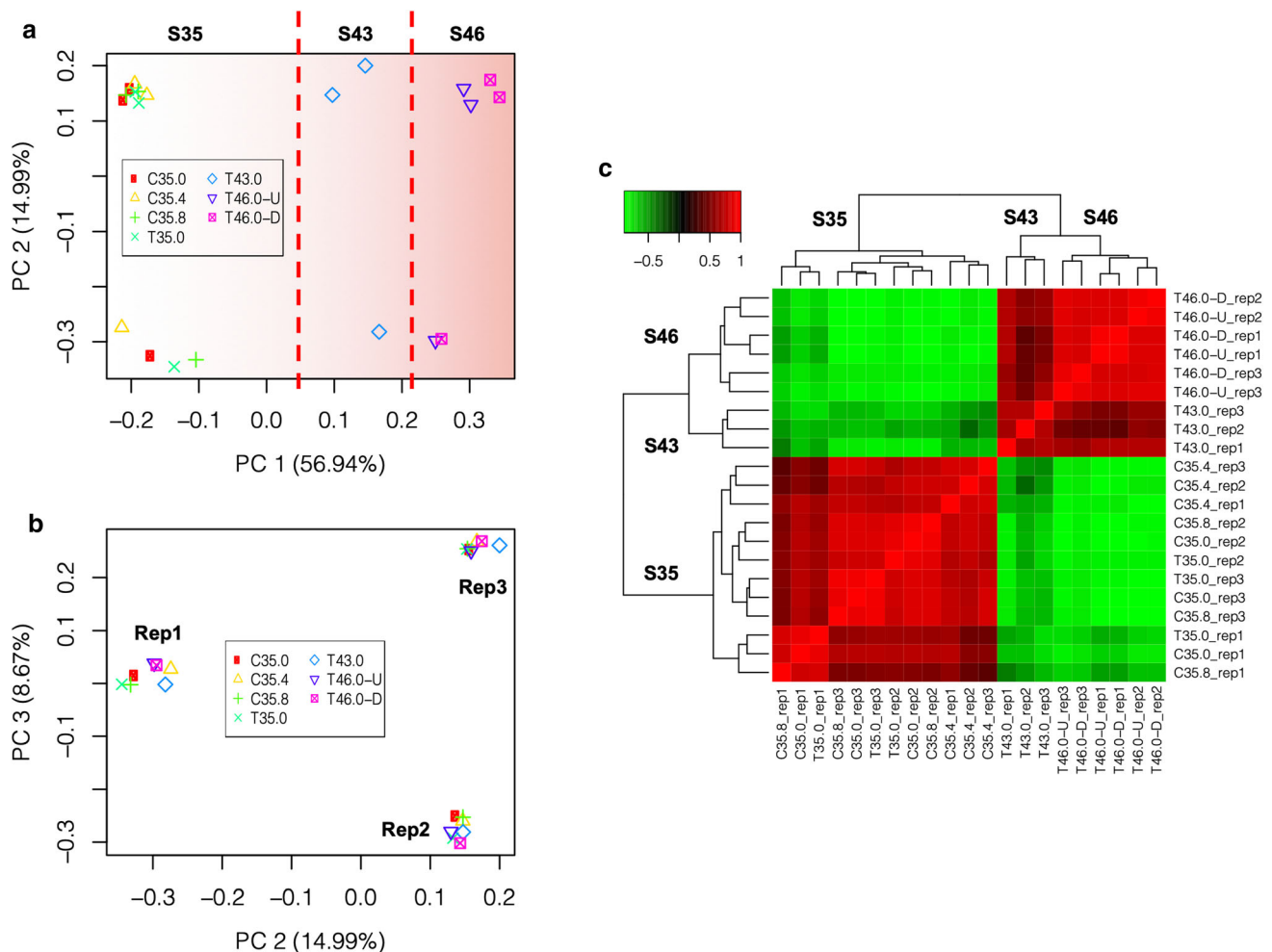


Fig. 2 Principal component analysis and gene expression heatmap. Principal component analysis was conducted based on 17,908 functionally annotated transcripts. PC1 is coincident with salinity gradient (highlighted with a red-to-white gradient) and clusters RNA-Seq libraries into three groups based on salinity levels (S35: C35.0, C35.4, C35.8, T35.0; S43: T43.0; S46: T46.0-U, T46.0-D) (a), within which variations between biological replicates (Rep) are reflected by PC2 and PC3 (b). A gene expression heatmap was plotted based on

4954 differentially expressed genes and shows the same clustering as in the principal component analysis (c). RNA-Seq libraries are labeled: T35.0: treatment group at 35.0‰ (0 h); T43.0: treatment group at 43.0‰ (12 h); T46.0-U: undetached coral tissues in treatment group at 46.0‰ (24 h); T46.0-D: detached polyps in treatment group at 46.0‰ (24 h); C35.0: control group at 35.0‰ (0 h); C35.4: control group at 35.4‰ (12 h); C35.8: control group at 35.8‰ (24 h)

Master Mix (Invitrogen, USA). Based on our transcriptomic data, we designed a qPCR assay to examine expression profiles of 10 stress genes during polyp bail-out induction (Table 1). Genes selected belong to the extrinsic apoptotic signaling pathway (FAS and CASP8), the fibroblast growth factor signaling pathway (FGF2 and FGFR2), the Ras signaling pathway (RHO, a Ras-like GTP-binding protein), or participate in proteolysis (MMP19 and MMP24), or in anti-apoptotic/survival signaling (JNK, NFKB1, and XIAP). A β -tubulin gene (TUBB) was included as an internal control gene for the qPCR analysis. For genes identified in multiple transcripts, the transcript with the highest expression level was selected as the representative in the qPCR assay, based on the assumption that such transcripts had the greatest biological

significance. For each qPCR reaction, 200 μ M of each primer and 1 μ L of synthesized cDNA (concentration undetermined) were mixed with iQ SYBR Green Supermix (Bio-Rad, USA) to a total volume of 10 μ L. Specificity of primer pairs was confirmed by a melting curve analysis (MCA) and qPCR efficiencies were tested in 10x-serial dilution with at least four concentration points ($N = 3$ at each concentration). All qPCR reactions were performed on a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, USA) under the following conditions: an initiation step at 95 $^{\circ}$ C for 3 min followed by 40 cycles of PCR amplification at 95 $^{\circ}$ C for 15 s and 60 $^{\circ}$ C for 30 s. For each gene in the qPCR assay, the mean cycle threshold (CT) value from two technical replicates of a given sample was employed to calculate the relative gene expression

Table 1 Primers used in the qPCR assay

Gene	UniProt ID	Primer (5' → 3')	Ref (Pos)	E (%)
FAS	TNR6_PIG	TNR6_F1: GCCAACAACTCAGGAGACAC TNR6_R1: GCAAAGCAATTACCGCAAAC	4317_c14_g1_i1 (617–828)	93.8
CASP8	CASP8_HUMAN	CASP8_F1: AATGAACACTTCCAGGGAAAC CASP8_R1: GGCTAAGCAAGCAGCAAAC	22697_c0_g1_i1 (380–587)	95.2
FGF2	FGF2_BOVIN	FGF2_F1: CTAAACCCACCAGAAAGTCATC FGF2_R1: TTGTTTCCAATGCCGTCC	28352_c0_g1_i1 (845–1031)	97.2
FGFR2	FGFR2_XENLA	FGFR2_F1: CCATCTTGGACAACACAAAAAC FGFR2_R1: TCCTCTGCTGACCTGATAAC	50055_c4_g1_i1 (540–762)	96.6
RHO	RHO_APLCA	RHO_F1: TCTGCCACGAGGAAAAAAC RHO_R1: GCCTGTCATAATCTTCTGTTC	53528_c27_g1_i1 (197–398)	93.7
MMP19	MMP19_HUMAN	MMP19_F1: GGTCTAAAGTAGAGACCCCAG MMP19_R1: CCAAACGAACATTGCGTCC	3229_c0_g1_i1 (19–200)	96.1
MMP24	MMP24_RAT	MMP24_F1: TCCAATGGAAGAACGGCAG MMP24_R1: TGAGTAAAGATGCCATGAAGAG	20183_c0_g1_i1 (90–308)	98.6
JNK	JNK_DROME	JNK_F1: TCATCAGAATCAGACAGATGATAAG JNK_R1: GAACTGCACGAATTGTTTCATTA	33455_c1_g1_i2 (847–1013)	92.0
XIAP	XIAP_HUMAN	XIAP_F1: TTCCAGTCAAAGACCCCAG XIAP_R1: TCGCCAGTACCCAAGTAATAG	30610_c1_g1_i1 (1637–1832)	93.1
NFKB1	NFKB1_CHICK	NFKB1_F1: CCTTTTGTACCAGTGCAGTG NFKB1_R1: TGTCATGAATCTGTTTAGAGGC	27043_c0_g1_i2 (259–449)	90.7
TUBB	TBB_PARLI	TUBB_F2: GCAGTTCACGGCTATGTTC TUBB_R2: TTTTCACCCTCCTCTTCCTC	36257_c3_g1_i1 (1427–1615)	98.4

The reference transcript (Ref), primer positions (Pos), and BLASTX-based annotation (UniProt ID) are indicated for each gene (GenBank assembly accession: GIDI00000000). Efficiency of qPCR (*E*) for each gene was tested in a 10x-serial dilution with at least four concentration points (*N* = 3 at each concentration)

($\Delta CT = CT_{\text{control gene}} - CT_{\text{target gene}}$). As qPCR results from both Experiments II and III showed the same trend of expression changes with only slight variation in magnitude (Fig. S1 and Table S2), data from the two experiments were pooled to increase statistical power. The ΔCT values of treatment group were normalized to those of the corresponding control group (on an average of six replicates) and to the initial conditions (35.0‰) to determine expression changes during the experiments ($\Delta\Delta CT = \Delta\Delta CT_{\text{given salinity level}} - \Delta\Delta CT_{\text{initial condition}}$; $\Delta\Delta CT = \Delta CT_{\text{treatment group}} - \Delta CT_{\text{control group}}$). Data are presented as means \pm SDs (standard deviations) for each gene, and statistical significance between time points was tested using Welch's ANOVA with a Games-Howell post hoc test.

Results

Polyp bail-out induction

To induce the polyp bail-out response in *Pocillopora acuta*, we established a hyperosmotic environment by gradual

addition of high-salinity seawater (48‰). Disregarding differences in lighting and water conditions among the three experiments in this study, the same salinity-change profiles and morphological changes were observed (Fig. 1). In the treatment group (Fig. 1, upper panels), tentacle retraction was observed when salinity increased from 40.0 to 43.0‰ (6–12 h). Coenosarc tissues started to thin/degrade after salinity reached 43.0‰ (12 h) and became more perceptible at 45.0‰ salinity (18 h). At 46.0‰ salinity (24 h), complete coenosarc degradation was observed at some part of the coral fragments and about 30–50% of the polyps were found detached from the skeletons, apparently intact. To examine viability of polyps after the bail-out response, in each experiment we transferred some detached polyps to the control tank immediately after their detachment. In all these polyps, viability was evidenced by rotatory movement after few hours of rescue, which could be maintained for over 24 h (supplementary video). For control groups in all the three experiments, salinity changes during the experiment period were < 1‰ and no sign of polyp bail-out or other stress response was observed during the experiments (Fig. 1, lower panels).

Transcriptome assembly and differential gene expression analysis

After filtering RNA reads from coral-associated microbiomes and removing transcripts of low coverage, 73,903 transcripts were reconstructed (N50 = 1278 nt; E90N50 = 1746 nt; GenBank assembly accession: GIDI00000000), with 84% transcriptomic completeness (fragmented, 9.7%; missing, 6.2%) identified by the BUSCO analysis. From the transcriptome assembly, 17,908 transcripts were functionally annotated as eukaryotic genes. Differential gene expression (DE) analysis identified 6827 differentially expressed genes (DEG) among the three pairwise comparisons (S35/S43, S35/S46, and S43/S46; Table 2), including 11 caspase-like genes and 22 matrix metalloproteinase (MMP)-like genes (Table S3). A Fas receptor (FAS)-like gene (Pacuta_4317_c14_g1_i1) was upregulated in our transcriptomic data, but was statistically insignificant in the DE analysis, due to large variation between biological replicates. A Fas ligand (FASL)-like gene with low coverage was found in the raw transcriptome assembly (Fig. S2) and was removed in the final version of the transcriptome assembly.

In the comparison S35/S43, 3473 DEGs were identified (Table 2), with 1951 showing upregulation (S43 > S35) and 1522 showing downregulation (S43 < S35). In S35/S46, 6483 DEGs were identified (Table 2), with 2874 and 3609 showing upregulation (S46 > S35) and downregulation (S46 < S35), respectively, while in the comparison S43/S46, 99 upregulated (S46 > S43) and 215 downregulated (S46 < S43) DEGs were identified (Table 2). Expression heatmap of the 6827 DEGs further showed three expression profiles that can roughly be characterized as mild upregulation (cluster 1; 2961 DEGs), downregulation (cluster 2; 3788 DEGs), and huge upregulation (cluster 3; 78 DEGs) (Fig. 3).

Table 2 Numbers of differentially expressed genes (DEG) among comparisons between S35, S43, and S46

	S35	S43
S43	1951 (up) 1522 (down)	
S46	2874 (up) 3609 (down)	99 (up) 215 (down)

A total of 6827 DEGs were identified among the comparisons. Numbers of DEGs with up- and downregulation (in comparison with condition at the top of each column) are indicated separately for each pairwise comparison

Gene ontology enrichment analysis

To identify molecular mechanisms involved in the polyp bail-out response, gene ontology (GO) enrichment analysis was conducted for DEGs identified in the DE analysis. As most enriched GO terms in our results are likely involved in general responses of corals to osmotic fluctuations, the discussion of which is beyond the scope of this study, we paid specific attention to biological processes and signaling pathways related to apoptosis, proteolysis, and cell survival, which have been proposed as participating directly in polyp bail-out (Kvitt et al. 2015; Wecker et al. 2018). Among upregulated DEGs (clusters 1 and 3), GO categories such as *protein ubiquitination*, *tumor necrosis factor (TNF)-mediated signaling pathway*, *positive regulation of I-kappaB kinase/NF-kappaB signaling*, *apoptotic process*, *Ras protein signal transduction*, *fibroblast growth factor (FGF) receptor signaling pathway*, *extrinsic apoptotic signaling pathway*, and *proteolysis* were significantly enriched ($p < 0.05$; Table 3). Among downregulated DEGs (cluster 2), significant enrichment was identified in several GO categories related to cellular metabolism and cell cycle, but no specific signaling pathway or apoptosis/proteolysis-related GO category showed significant enrichment.

Quantitative PCR assay

To achieve finer temporal resolution of genetic analysis and to validate genetic responses identified in our transcriptomic data, we conducted two additional polyp bail-out experiments (Experiments II and III) and collected RNA samples at 6-h intervals for quantitative PCR (qPCR) analysis. For the 10 stress genes in our qPCR assay, gene expression changes showed trends concordant with those identified in the transcriptomic analysis and three expression profiles were consistently identified in both Experiments II and III, despite variations in lighting and water conditions (Fig. S1 and Table S2). For the CASP8 and FAS genes, significant upregulation was identified when salinity increased from 35.0 to 43.0‰ (3.3- and 10.8-fold changes, respectively; Table S2), after which expression remained statistically unchanged (Fig. 4, Profile I). A second expression profile was exhibited by the FGF2, FGFR2, RHO, and the two MMPs genes. For these five genes, little upregulation (related to the magnitudes along the whole experiments) was identified at 35.0–40.0‰ salinity (1.6–11-fold changes; Table S2), but was followed by remarkable upregulation from 40.0 to 45.0‰ (20–1167-fold changes; Table S2), at which salinity upregulation reached its peak (Fig. 4, Profile II). In contrast, for the NFKB1, JNK, and XIAP genes, linear upregulation was detected starting from different salinity levels

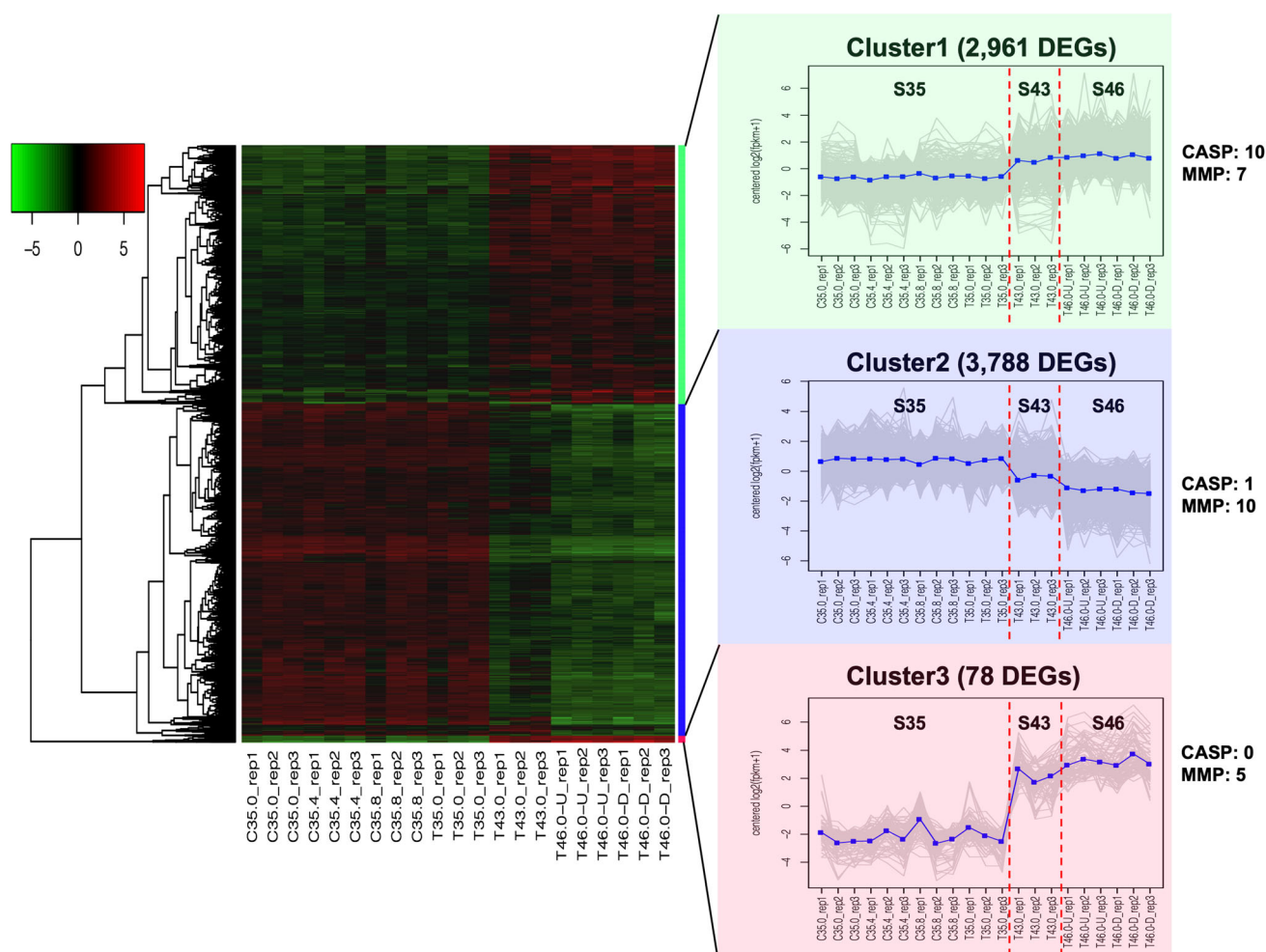


Fig. 3 Differential gene expression analysis for comparisons among S35, S43, and S46. A gene expression heatmap groups 6827 differentially expressed genes (DEG) into three clusters: cluster 1—mild upregulation along the salinity gradient; cluster 2—mild

(35.0–43.0‰) and peaked at the endpoint salinity (46.0‰) of the experiments (Fig. 4, Profile III).

Discussion

Hyperosmosis induces polyp bail-out in pocilloporid corals

In three pocilloporid corals, i.e., *Pocillopora damicornis*, *Seriatopora hystrix*, and *Stylophora pistillata*, Shapiro et al. (2016) first showed that polyp bail-out can be induced by evaporation-driven hyperosmotic stress. In the present study, we applied hyperosmotic stress to *P. acuta* by addition of hypersaline artificial seawater and observed polyp bail-out when seawater salinity reached 46.0‰ after 24 h. Additive salinity increases differ from evaporation-based salinity increases in that the rate of salinity change

decreases during the treatment period; and cluster 3—huge upregulation along the salinity gradient. Numbers of caspase-like (CASP) and matrix metalloproteinase-like (MMP) genes in each cluster are indicated

decreases during the treatment period. This slowdown of stress elevation was reflected in our transcriptomic data, which showed 3473 DEGs in the early stage of experiments (35.0–43.0‰) but only 314 DEGs in the later stage (43.0–46.0‰). Despite this difference in the salinity-change profiles between the present study and Shapiro et al. (2016), polyp bail-out was observed in both studies with the same process and at relatively similar stress levels (11–15% higher than ambient conditions). This consistency implies that polyp bail-out may be a common response to extreme hyperosmosis in pocilloporid corals from different habitats. Since detached polyps are viable after hyperosmosis-induced polyp bail-out and are able to resettle once stress abates (Shapiro et al. 2016; Liu et al. 2020), this stress response may represent a mechanism for coral polyps to escape and survive temporary lethal stresses (Sammarco 1982). Additional studies, however, are needed

Table 3 Selected significantly enriched gene ontology categories among differentially expressed genes (DEGs) during induction of polyp bail-out

GO category	Count	<i>p</i> value	FE
<i>Cluster 1 (2961 DEGs)</i>			
Protein ubiquitination	111	2.03E−05	1.56
MAPK cascade	34	2.56E−04	1.97
Tumor necrosis factor-mediated signaling pathway	20	1.70E−03	2.70
Positive regulation of I-kappaB kinase/NF-kappaB signaling	43	1.81E−03	1.75
Apoptotic process	102	2.10E−03	1.35
JNK cascade	15	2.67E−03	2.58
Response to oxidative stress	44	5.41E−03	1.67
Ras protein signal transduction	16	2.29E−02	1.95
Fibroblast growth factor receptor signaling pathway	24	2.74E−02	1.72
Extrinsic apoptotic signaling pathway	9	3.13E−02	2.67
Negative regulation of extrinsic apoptotic signaling pathway via death domain receptors	10	3.40E−02	2.97
<i>Cluster 2 (3788 DEGs)</i>			
Oxidation–reduction process	215	3.93E−08	1.43
DNA replication	59	2.06E−05	1.72
DNA repair	102	1.12E−03	1.36
Cell division	101	4.48E−03	1.29
Cell cycle	95	7.57E−03	1.28
<i>Cluster 3 (78 DEGs)</i>			
Proteolysis	8	3.34E−03	4.00

Total number of DEGs in each cluster is indicated. Number of DEGs (count), *p* values, and fold enrichment (FE) are indicated for each GO category

to further illuminate the ecological significance of this stress response.

TNF signaling leads to apoptosis and coenosarc degradation

The extrinsic apoptotic signaling pathway, comprising signals mediated by the TNF receptor family and a caspase cascade initiated by caspase-8, is a highly conserved pathway of programmed cell death among animals (Quistad et al. 2014). In the present study, the significant enrichment of GO terms *apoptotic process* and *extrinsic apoptotic signaling pathway*, as well as the coincident upregulation of the FAS and CASP8 genes identified in our genetic analyses, supports the hypothesis of apoptosis-mediated polyp bail-out proposed in previous studies (Kvitt et al. 2015; Wecker et al. 2018). Interestingly, in the present study, overexpression of both the CASP8 and FAS genes peaked at 43.0‰ and remained stable afterward, even through the hyperosmotic stress continued to increase

(from 43.0 to 46.0‰ salinity). This plateau of gene expression is coincident with coenosarc thinning/degradation in our morphological observations. Our results thus indicate that corals are able to moderate the apoptotic response once it reaches an appropriate level, adding further support to the hypothesis of “polyp-controlled programmed cell death” in the coenosarc-degradation stage in polyp bail-out (Kvitt et al. 2015).

Interestingly, even with significant enrichment of the TNF signaling pathway and upregulation of the FAS gene in our genetic data, no overexpression of TNF was found during polyp bail-out induction in this study. Similar to our finding, in Wecker et al. (2018) TNF exhibited an expression change opposite to that of caspase and TNF receptor genes during the coenosarc-degradation stage. These observations imply that apoptosis in polyp bail-out is probably disentangled from coral TNF signals. Considering the ancient origin of core apoptotic mechanisms and the growing number of reports on inter-kingdom communication between microbes and their eukaryotic hosts (Hughes and Sperandio 2008; Segovia 2008; Pacheco and Sperandio 2009), we hypothesize that the triggering signal of apoptosis in polyp bail-out is derived from microorganisms associated with corals (Fig. 5). Changing of culture environments can substantially alter the composition of coral-associated microbiomes and the chemical signals provided from microbes to the coral animals (Littman et al. 2011; Sharp and Ritchie 2012; Webster et al. 2013). These coral-associated microorganisms therefore may play an intermediate role between environmental stresses and coral polyp bail-out. In fact, participation of microorganisms in polyp bail-out has recently been demonstrated with induction of the bail-out response in *P. damicornis* via inoculation with a coral pathogen, *Vibrio coralliilyticus* (Gavish et al. 2018). Nevertheless, since the TNF signaling pathway is believed to have originated with the Metazoa (Wiens and Glenney 2011; Quistad and Traylor-Knowles 2016) and inter-kingdom communication can proceed via non-coding RNA (Leitão and Enguita 2016; Bayer-Santos et al. 2017), a microbe-derived molecule triggering the coral TNF signaling pathway cannot be identified with the methods used in this study, even in the raw dataset with *Symbiodinium*/microbiome sequences. Further studies are therefore required to verify this cross-talk and to identify the key signaling molecule that activates the extrinsic apoptotic signaling pathway in coral polyp bail-out.

FGF signaling induces ECM degradation and polyp detachment

A previous study of *P. damicornis* suggested participation of ECM degradation in the polyp-detachment step of bail-out response (Wecker et al. 2018). Congruent with that

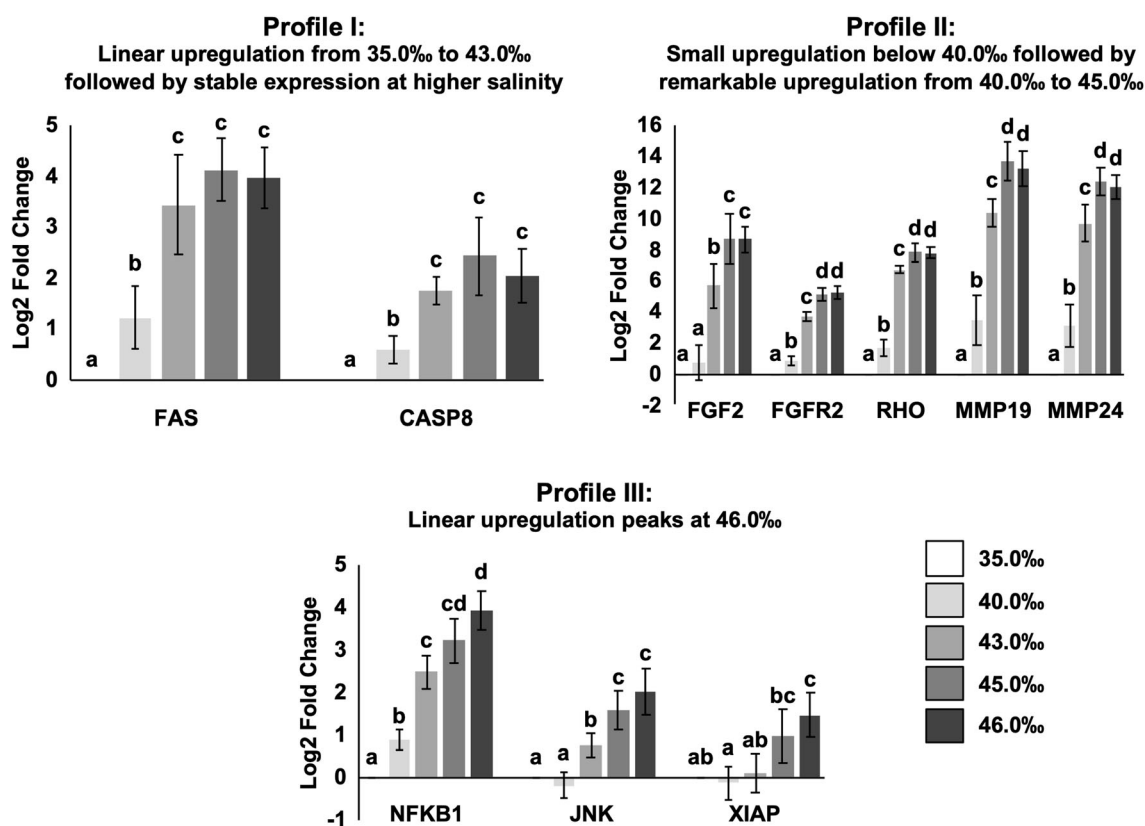


Fig. 4 Gene expression profiles of 10 stress genes during polyp bail-out induction. The FAS and CASP8 genes show significant upregulation during salinity increases from 35.0 to 43.0‰ and remain statistically stable at salinity levels of 43.0–46.0‰ (Profile I). The FGF2, FGF2, RHO, MMP19, and MMP24 genes display a common expression profile that shows little upregulation from 35.0 to 40.0‰, followed by remarkable upregulation from 40.0 to 45.0‰, after which

expression stabilizes (Profile II). For the JNK, NFKB1, and XIAP genes, linear upregulation was observed peaking at 46.0‰ salinity (Profile III). Data are labeled as the salinity levels of treatment group at the sampling points. Statistical analysis was conducted using Welch's ANOVA with a Games-Howell post hoc test. Groups with significant differences ($p < 0.05$) are indicated. Data are log₂-transformed and are expressed as means \pm SDs (standard deviations)

study, we found significant enrichment of *proteolysis* and remarkable expression changes of several MMP-encoding transcripts during polyp bail-out induction. MMPs are a family of proteases that function in ECM degradation (Birkedal-Hansen et al. 1993; Kojima et al. 2000). Regulation of these MMPs in polyp bail-out is probably linked to the FGF and Ras signaling pathways, based on (1) the concurrent enrichment of GO categories *FGF receptor signaling pathway* and *Ras protein signal transduction* in our transcriptomic data, and (2) matched expression profiles between the FGF2, FGFR2, RHO, and the two MMP genes in our qPCR analysis. In many mammalian cells, the FGF-Ras-MMP signaling pathway is involved in cell migration, angiogenesis, and cancer metastasis (Liu et al. 2002; Pintucci et al. 2003; Krejci et al. 2005). Since upon activation, some MMPs also induce release of FGF2 (Whitelock et al. 1996), this feedback circuit between MMPs and FGF signals may explain the remarkable upregulation of FGF2 and MMPs at 40.0–45.0‰ in our qPCR analysis, and the irreversibility of coral polyp bail-out. Accordingly, we hypothesize that in polyp bail-out,

FGF signaling is likely relayed by the Ras signaling pathway to activate MMPs that initiate subsequent ECM degradation and polyp detachment (Fig. 5).

Survival mechanisms of polyps during the bail-out response

Based on histological data, Kvitt et al. (2015) demonstrated that the apoptotic response in polyp bail-out occurs mostly in the coenosarc and adjacent tissues, but not in polyps. However, in our transcriptomic data, no significant difference in caspase gene expression was found between coenosarc-containing coral tissues (T46.0-U) and detached solitary polyps (T46.0-D). We therefore hypothesize that tissue-specific apoptosis in polyp bail-out is mediated at the posttranscriptional level. In support of this hypothesis, in our transcriptomic data we identified significant enrichment of GO category *protein ubiquitination* and upregulation of the XIAP gene, which have both been shown to regulate caspase activity and cell death (Deveraux et al. 1997; Bader and Steller 2009; Chen and Qiu 2013). It has

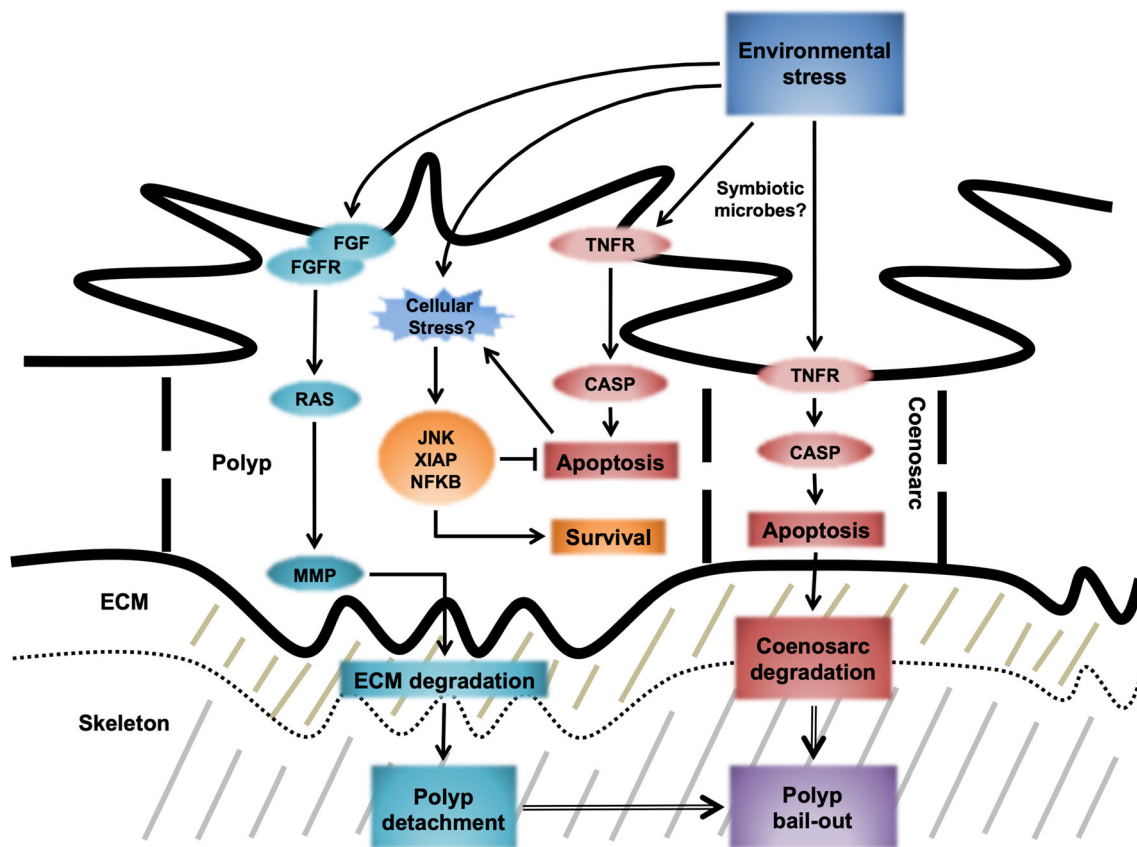


Fig. 5 Putative signaling pathways in polyp bail-out. Environmental stress triggers the TNF and FGF signaling pathways, which activate the caspase (CASP) and matrix metalloproteinase (MMP) cascades, respectively. Activation of caspases leads to apoptosis in the coenosarc and to its subsequent degradation, while activation of

MMPs, likely through the Ras signaling pathway (RAS), results in extracellular matrix (ECM) degradation and detachment of polyps. In polyp bodies, anti-apoptotic and cell survival signals mediated by genes, such as JNK, NFKB, and XIAP, are activated to suppress the apoptotic response, which in turn promotes polyp survival

been proposed that an anti-apoptotic response is activated by TNF signals at a later stage of polyp bail-out, based on the coincident upregulation of TNF genes and downregulation of caspases and TNFRs after coenosarc degradation (Wecker et al. 2018). Similarly, in the present study, enrichment of GO terms such as *JNK cascade*, *negative regulation of extrinsic apoptotic signaling pathway via death domain receptors*, and *positive regulation of I-kappaB kinase/NF-kappaB signaling* was identified among upregulated DEGs, indicating activation of anti-apoptotic/cell survival responses during polyp bail-out (Van Antwerp et al. 1996; Roulston et al. 1998; Karin and Lin 2002). These responses, however, were not accompanied by TNF signals, as our transcriptomic analysis showed no TNF overexpression during the experiment, even in detached polyps (T46.0-D). Alternatively, survival and anti-apoptotic signals may be associated with cellular stresses accumulated during polyp bail-out, implied by the significant enrichment of GO category *response to oxidative stress* and by linear upregulation of survival and anti-apoptotic genes along salinity increases in our qPCR

experiments. Accordingly, our results suggest that the tissue-specificity of apoptosis in polyp bail-out may be attributed to spatial variation in anti-apoptotic signals, leading to inhibition of caspase activity in coral polyps and to their subsequent survival (Fig. 5). Identifying the ultimate trigger of these anti-apoptotic/cell survival signals and their functional involvement in polyp bail-out, however, requires further investigation.

New insights distinguish polyp bail-out and coral bleaching

Based on our results, it appears that the TNF receptor-mediated extrinsic apoptotic pathway, FGF-mediated ECM degradation, and anti-apoptotic/survival signals are involved in polyp bail-out (Fig. 5). Bail-out is distinct from coral bleaching, which is generally thought to be mediated via the intrinsic apoptotic pathway triggered by oxidative stress from dinoflagellate symbionts (Weis 2008; Kvitt et al. 2011). The unique activation of FGF-mediated ECM degradation in polyp bail-out further distinguishes it from

bleaching with respect to morphological consequences. In polyp bail-out, free-living polyps are generated while in bleaching, azooxanthellate coral polyps remain in situ after stress. As some environmental stresses, such as acidification and thermal stress, are common inducers for both responses, understanding mechanisms by which corals determine which stress response is to be employed, is essential to understand the relative ecological significance of polyp bail-out versus coral bleaching.

Future applications of polyp bail-out

Beside its distinctness from coral bleaching, the discreteness of pathways leading to apoptosis and ECM degradation in bail-out suggests possible independence of coenosarc degradation and polyp detachment. Indeed, separation of these processes has been reported in previous studies using different treatments (Domart-Coulon et al. 2004; Kvitt et al. 2015; Fordyce et al. 2017) and was observed in our preliminary experiments under different levels of hyperosmotic stresses (Fig. S3). Also, since detachment of polyps is mediated by degradation of ECM, we hypothesize that it may be possible to induce polyp resettlement either by stimulating ECM secretion or by providing suitable ECM components to bailed-out polyps. In support of our hypothesis, previous studies have found that fibronectin-like peptide facilitated attachment of solitary polyps to resettlement substrates (Domart-Coulon et al. 2004; Puverel et al. 2005). However, in both Domart-Coulon et al. (2004) and Puverel et al. (2005), coral polyps tended to dissociate into single cells after the resettlement. This cell dissociation was likely caused by calcium-deprivation treatments used to detached polyps in both studies, which might be expected to impact cell–cell adhesion (Hobmayer et al. 2001; Marshall and Clode 2004; Van Roy and Berx 2008). In contrast, hyperosmosis-based polyp bail-out induction offers advantages over Ca-deprivation in maintaining morphological integrity of polyps after their resettlement (Shapiro et al. 2016; Liu et al. 2020). However, the extreme hyperosmotic stress applied during polyp bail-out induction could impair coral health and reduce resettlement success of detached polyps (Shapiro et al. 2016). Our identification of signaling pathways underlying the bail-out response sheds light on development of alternative methods for polyp bail-out induction, such as using ligands or agonists specifically targeting these pathways. By minimizing systematic stress and avoiding undesirable responses in corals, new methods are expected to facilitate acquisition of resettled polyps that are morphologically and physiologically intact. Given that polyp bail-out from a small coral fragment can generate tens to hundreds of individual polyps (Sammarco 1982; Shapiro et al. 2016; Fordyce et al. 2017), this coral propagation method

provides potentially significant advantages over the commonly used cutting/fragmentation strategy in regard to the output quantity. Improvement of success rates in resettlement and survival of bailed-out polyps will potentiate their use for mass production of coral colonies for reef restoration. Furthermore, since polyp bail-out may greatly influence local recruitment in a variety of coral species (Fordyce et al. 2017), our findings provide a genetic perspective for future studies to understand how this response shapes coral reef ecosystems in shallow waters.

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Data accessibility Data supporting the findings of this study are available on the NCBI database with the following accession numbers: RNA-Seq raw data: NCBI SRA: SRR10696823-SRR10696829, SRR10708182-SRR10708188, SRR10708225-SRR10708230. Transcriptome assembly: GenBank assembly accession GIDI00000000.

Compliance with ethical standards

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

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