Received: 27 April 2021 | Revised: 15 October 2021 | Accepted: 20 October 2021

DOI: 10.1111/mec.16259

ORIGINAL ARTICLE

MOLECULAR ECOLOGY WILEY

Hypoxia as a physiological cue and pathological stress for coral larvae

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Funding information
Australian Research Council, Grant/Award Number: DP180100074; Deutsche Forschungsgemeinschaft, Grant/Award Number: 433042944 and 458901010; Gordon and Betty Moore Foundation, Grant/Award Number: GBMF9206; Small Business Innovation Research, Grant/Award Number: TF6.7.1

Abstract

Ocean deoxygenation events are intensifying worldwide and can rapidly drive adult corals into a state of metabolic crisis and bleaching-induced mortality, but whether coral larvae are subject to similar stress remains untested. We experimentally exposed apo-symbiotic coral larvae of *Acropora selago* to deoxygenation stress with subsequent reoxygenation aligned to their night-day light cycle, and followed their gene expression using RNA-Seq. After 12 h of deoxygenation stress (~2 mg O_2/L), coral planulae demonstrated a low expression of HIF-targeted hypoxia response genes concomitant with a significantly high expression of *PHD2* (a promoter of *HIFα* proteasomal degradation), similar to corresponding adult corals. Despite exhibiting a consistent swimming phenotype compared to control samples, the differential gene expression observed in planulae exposed to deoxygenation-reoxygenation suggests a disruption of pathways involved in developmental regulation, mitochondrial activity, lipid metabolism, and O_2-sensitive epigenetic regulators. Importantly, we found that treated larvae exhibited a disruption in the expression of conserved HIF-targeted developmental regulators, for example, Homeobox (*HOX*) genes, corroborating how changes in external oxygen levels can affect animal development. We discuss how the observed deoxygenation responses may be indicative of a possible acclimation response or alternatively may imply negative latent impacts for coral larval fitness.

KEYWORDS
coral, development, gene expression profiling, hypoxia, RNA-Seq

1 | INTRODUCTION

Ocean oxygen content is declining worldwide as our climate warms and coastal pollution accelerates (Breitburg et al., 2018; Schmidtko et al., 2017). Some tropical regions, including the Central Pacific and the Indian Ocean, have lost up to 40% of their dissolved oxygen content in the last 50 years, making ocean deoxygenation the most dramatically changed ecologically relevant factor in the marine environment (Schmidtko et al., 2017). Until recently, the extent and nature of ocean oxygen loss for marine life in coral reefs had been almost entirely neglected (Hughes et al., 2020; Nelson & Altieri, 2019). Underreported, but intensifying, deoxygenation events have resulted in large scale mortality of reef biota (Altieri et al., 2017). In such cases the oxygen supply for the organism drops to levels insufficient to sustain "normal" functioning, that is, entering a hypoxic state. Recent experiments have confirmed such mortality events whereby deoxygenation events rapidly drive adult corals into a state of metabolic crisis that manifests as bleaching-induced mortality (Alderdice et al., 2020). It remains unknown whether and how deoxygenation drives a metabolic crisis for coral larvae in a similar manner. However, it is unlikely that findings recently reported for adult corals (Alderdice et al., 2020) can directly transfer to coral larvae that exhibit very different physiologies related to their predominant free-living planktonic versus benthic stages. Furthermore, larvae of broadcast spawning corals are initially apo-symbiotic, that is, without their photosynthetic algal symbionts (Harrison & Wallace, 1990) that normally contribute to diel hyperoxia-hypoxia fluctuations in coral tissues (Kühl et al., 1995).

Most corals broadcast their gametes into the water column for embryogenesis and larval development to take place within the pelagic zone (Harrison, 2011; Harrison & Wallace, 1990; Ritson-Williams et al., 2009), where oxygen is more readily available compared to the benthos where adult corals reside. Planktonic coral larvae are usually competent to undergo benthic settlement and metamorphosis into a juvenile coral after 4–8 days of development in waters under normoxia (Harrison, 2006; Jones et al., 2009; Portune et al., 2010; Reyes-Bermudez et al., 2009; Szmant & Miller, 2006; Wilson & Harrison, 1998). Nevertheless, longer planktonic life stages up to several months can occur. For instance, although 20% of larvae of *Platygyra daedalea* settled after ~4 days, this species demonstrated a maximum larval settlement-competency period of ~100 days after spawning (Connolly & Baird, 2010; Graham et al., 2008; Nozawa & Harrison, 2002). Whilst the dominant larval life phase is in pelagic waters at oxygen saturation levels of 6–8 mg O_2/L (normoxia), evidence may suggest a capacity for sustained low O_2 tolerance. Firstly, coral groups with non-feeding larvae for which extremely long pelagic durations have been documented (Connolly & Baird, 2010; Graham et al., 2008; Nozawa & Harrison, 2002, 2005) exhibited a sustained hypometabolic state. More specifically, a hypometabolic state sustained following the bioenergetic reprogramming at ~4 days post-fertilisation whereby a rapid decline in O_2 consumption and lipid metabolism occurred (Graham et al., 2013). Secondly, once at the benthic environment for settlement, coral larvae exhibit increasing O_2 limitation due to the diffusive boundary layer of the benthic substratum where oxygen availability is lower in the absence of photosynthesis (Jørgensen & Revsbech, 1985). However, the molecular mechanisms involved in such metabolic adjustments are unresolved, and whether they equip coral larvae to withstand hypoxic conditions remains unknown.

Animal cells under hypoxia typically activate an extensive cohort of genes to ensure O_2 supply matches the metabolic, bioenergetic, and redox demands (Kaelin & Ratcliffe, 2008). In doing so, cells ultimately reduce their mitochondrial activity, shift to anaerobic energy production, induce lipid reorganisation, and secrete defence molecules such as antioxidants (Loenarz et al., 2011). These mechanisms are also employed by apo-symbiotic deep-sea corals that appear to live under very low O_2 conditions of 1–2 mg O_2/L in the Red Sea (Roder et al., 2013; Yum et al., 2017). Coordination of such cellular reprogramming in most metazoans is governed by the highly conserved hypoxia-inducible factor (HIF) transcription factor (Kaelin & Ratcliffe, 2008). The constitutively expressed *HIFα* subunits are directly targeted for proteasomal degradation by prolyl hydroxylases under normoxia, but stabilized under limiting oxygen conditions, when they translocate to the nucleus to form an "active HIF complex" and induce expression of hypoxia-responsive genes (Rytkönen et al., 2011; Taabazuing et al., 2014). A complete HIF-associated hypoxia response system (HRS) was recently identified for adult corals of different *Acropora* species (Alderdice et al., 2020). This study also highlighted the important roles that heat shock protein (HSP) 90 can play in managing proteotoxic stress under hypoxic conditions (Jayaprakash et al., 2015), by stabilising *HIFα* proteins and inducing conformational changes to its structure critical for coupling with HIFβ to form the "active HIF complex" (Gradin et al., 1996; Hur et al., 2002; Isaacs et al., 2002; Katschinski et al., 2004; Minet et al., 1999). However, it is unknown whether coral larval stages employ this HIF gene network under hypoxia. Importantly, hypoxic microenvironments occur naturally in both the developing embryo and adult phases of mammals, and create specific niches that regulate cell stemness (Maltepe & Simon, 1998; Semenza, 2012; Simon & Keith, 2008). Consequently, the HIF gene system is also involved in targeting genes that function as important early development regulators for cell differentiation and proliferation, such as Homeobox (HOX) and Sonic hedgehog (SHH) genes (Bijlsma et al., 2009; Chen et al., 2015; Cowden Dahl et al., 2005; Downes et al., 2018; Koh & Powis, 2012). Understanding hypoxic cues for key developmental pathways in coral larvae is therefore as important as for metabolic stress management in healthy larval development.

Molecular regulatory pathways for cnidarian differentiation and morphogenesis is of general interest given how cnidarians can serve as a model for early metazoan development (Ball et al., 2004). Components of the Brachyury, Notch, and Wnt signalling pathways have gained the most focus so far in cnidarian developmental biology, including in coral (Ball et al., 2004; Marlow et al., 2012; Technau & Steele, 2011). Despite the signalling cross-overs between Notch/Wnt and HIF gene pathways (Gustafsson et al., 2005), the HIF system has yet to be explored in association with coral early development regulation. Similarly, the hairy and enhancer of split (*HES*), a metabolic transcriptional suppressor and a downstream target of

NOTCH and *HIF* (Downes et al., 2018), has so far only been discussed in adult corals in association with diel oscillations and light stress (Ottaviani et al., 2020; Ruiz-Jones & Palumbi, 2015). Interestingly, it is also known to function as a metabolic switch during development in *Drosophila melanogaster* (Zhou et al., 2008). Notably, in multicellular organisms, the ability to regulate reproduction, development, and nutrient utilization coincided with the evolution of nuclear receptors (NRs), transcription factors that utilize lipophilic ligands to mediate their function (Bookout et al., 2006). In particular, the nuclear receptor, Estrogen related receptor (*ESRRG*), is known to play a role in the cross-talk between metabolic capacity and activating developmental processes through the physical interaction with *HIFα* (Ao et al., 2008; Huss et al., 2015; Kumar & Mendelson, 2011; Li et al., 2013; Tennessen et al., 2011; Zou et al., 2014). Some aquatic species, such as zebrafish, are known to possess an HIF gene system and tolerate complete anoxia during development with no apparent adverse effects (Mendelsohn et al., 2008; Padilla & Roth, 2001; Pelster & Egg, 2018) (Mendelsohn et al., 2008; Padilla & Roth, 2001; Pelster & Egg, 2018), therefore raising the possibility that coral larvae development could be unaffected by hypoxic conditions.

This study builds on our recent study of the gene network involved in adult coral hypoxia stress responses (Alderdice et al., 2020), where we employed RNA-Seq to analyse the HIF gene system responding to deoxygenation-driven hypoxia as a pathological stress, that is, a level of stress that exceeds the host's ability to cope by physiological means. Here we assess whether an earlier life history stage of a coral is more vulnerable to environmental stress compared to their adult counterparts by exposing apo-symbiotic planula larvae of deoxygenation-susceptible parents (*Acropora selago*) to an experimental deoxygenation-reoxygenation regime aligned to their night-day cycle.

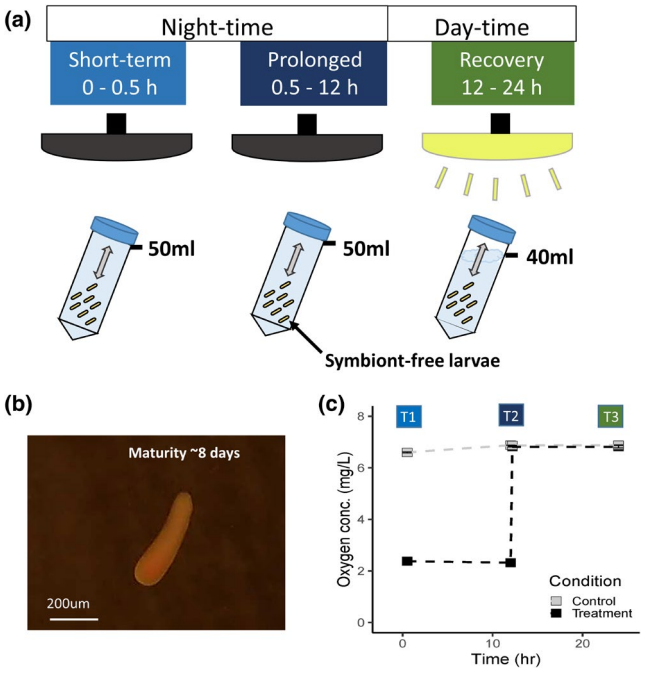


FIGURE 1 Details of experimental incubation setup. (a) Sampling conditions indicated for time points (T1–3) with short-term (0.5 h, T1) and prolonged (0.5–12 h, T2) deoxygenation stress in dark conditions during the night hours within a closed system followed by 12 h of reoxygenation (T3) in light conditions during the daytime within a closed system but with an air bubble introduced. (b) Microscope image of *Acropora selago* planula larvae used in experiment. (c) Oxygen concentration (mg O_2/L) levels across experiment for treatment and control settings. Symbols with error bars denote means ± standard error with $n = 4$ for each condition group

2 | MATERIALS AND METHODS

2.1 | Coral collection and larvae culture

Twenty gravid colonies of the reef-building coral species, *Acropora selago*, were collected from Vlasoff Reef in the northern Great Barrier Reef (GBR) on 23rd and 24th November 2018, coinciding with the full moon on the 23rd. Prior to collection, colonies were sampled by carefully breaking several branches to determine the presence of mature oocytes, as indicated by their pink colouration (Harrison et al., 1984). Gravid colonies were carefully transported in seawater to an aquaria facility for ex situ spawning and gamete collection. Colonies were maintained in a shaded outdoor closed-circulation aquarium (James Cook University, Cairns; 22–40°C min-max daily air temperature range, average water temperature of 28°C, salinity at 34 PSU and dissolved oxygen (DO) of ~6 mg O_2/L maintained via continuous aeration) and were monitored periodically at night to check for settling and spawning behaviours (Babcock et al., 1986). Colonies spawned on the third night after the full moon between 19:30 h and 20:00 h on 26th November 2018. Coral egg-sperm bundles were skimmed off the water surface and transferred to a fertilisation container. To maintain healthy water quality and prevent polyspermy, seawater containing excess sperm was siphoned off beneath the floating eggs and new filtered seawater was slowly added (dela Cruz & Harrison, 2020; Willis et al., 1997). Subsamples of embryos and eggs were then collected and examined under a stereomicroscope to determine percentage fertilization. Developing embryos were transferred into large rearing tanks (each containing 450 L of seawater) and larvae were maintained in healthy conditions by daily aeration and seawater renewal under a natural night day cycle. Absence of algal symbionts were confirmed via microscopy. For this experiment, apo-symbiotic planula larvae eight days after fertilisation were used, see Figure 1 for microscope image of example larvae used. Of note, *A. selago* larvae have been reported in other studies to reach competence from day 4 (Suzuki & Hayashibara, 2011). Therefore, our experimental larvae were at a life stage that would encounter low O_2 levels while searching to settle on benthic substrate that their adult forms inhabit.

2.2 | Experimental setup

To allow for comparison between coral larvae and adult life stages, the experimental design was analogous to the one used in a study

with adult counterparts, as described in Alderdice et al. (2020) but scaled down to accommodate for the much smaller larval size (Figure 1). Larval incubation conditions were established to mimic in situ reef conditions from where corals were sourced and acclimated during rearing, with the exception of the DO concentration, shown in Figure 1. Deoxygenation was applied as previously described (Alderdice et al., 2020) and, in brief, consisted of flushing seawater with N_2 down to ~2 mg O_2/L prior to additional flushing with CO_2 to account for subsequent increase in pH as per Klein et al. (2017). Seawater in the aliquoted larvae falcon tubes was drained and the prepared deoxygenated seawater was poured gently into the falcon tubes to the top to avoid air bubbles/spaces. For consistency, despite the lack of algal symbiont associated night-time intra-tissue hypoxia in the larvae, deoxygenation stress treatment was aligned to the night cycle to take into consideration potential circadian regulation of hypoxia genes during the night, prior to symbiotic algae acquisition. The incubation setup consisted of 24 x 50 ml transparent falcon tubes (12 for each of control and treatment) placed horizontally on a CO-Z orbital shaker (speed of 80 rpm) to prevent larval aggregation at the bottom of the tube and maintained in a temperature-controlled laboratory at 28°C. To prevent build-up of waste products in the seawater over time, we utilised a large water volume to biomass ratio (Camp et al., 2015), with 30–40 larvae per falcon tube. A photon scalar irradiance (PAR, 400–700 nm) of ~180 μmol photons/m^2/s was provided by Hydra52 LEDs on a 12:12 h light cycle (with a 4 h programmed ramping phase) and measured with a calibrated underwater scalar irradiance sensor (LiCor LI-193) connected to a light meter (LiCor Li-250A). Dark conditions were created by black-out plastic sheet placed over the incubation vessels throughout the 12 h night period. The low oxygen night-time phase was followed by a "recovery phase" of 12 h in LED-lit conditions and normoxia (~6 mg O_2/L). Falcon tubes were always closed and lids were sealed with parafilm, but in the "recovery phase" where 5 ml of water was poured off and air was introduced forming a headspace for continued oxygenation of the seawater (see Table S1, S2 for consistency of O_2 conditions in recovery phase for both control and treatment chambers).

Similar to the sampling collection for adults (Alderdice et al., 2020), larval samples were collected 0.5 h into (T1) and at the end (12 h, T2) of the deoxygenation night-time phase, and finally after 12 h re-exposure to light and normoxia (T3). Four falcon tubes (representative of the genetically diverse larvae produced via cross-breeding) from both treatment and control settings were removed at each time point T1–T3. Coral planulae were filtered (mesh size 280 μm) from the falcon tubes for each sampling time point with minimal water into Eppendorf tubes (500 μl) and filled with RNAlater (Thermo Fisher Scientific). After 0.5 h, the RNAlater was carefully removed and refreshed to make sure there was minimal water in the sample for sufficient preservation for subsequent molecular analysis. Additional samples at the onset of the experiment (Time zero, "T0") were not used given how the large transcriptional changes due to development over time would greatly influence comparative analysis with subsequent time points (Siboni et al., 2014). Rather, in this study we were most interested in the transcriptional changes due to the difference between hypoxia and normoxia, not in the difference between time points. Since there are no treatment samples for T0, we were not able to disentangle differences in gene expression due to development and experimental condition. Further, each sample ($n = 4$) consisted of 30–40 pooled larvae. As such, we considered the averaged gene expression of many individual larvae to avoid discrepant gene expression caused by "outlier" larvae. Further details of the experimental design and corresponding measured O_2 and pH under the treatment and control settings across time points are provided in Figure S1 and Table S1, S2. Note the small difference in pH of ~0.5 between treatment and control, due to adjusting seawater oxygen levels using N_2 and CO_2 bubbling. Such difference is within the natural diel cycle range of pH experienced by corals on reefs (Anthony et al., 2008; Cyronak et al., 2020) and was hence not specifically considered.

Coral planulae in treatment and control samples were visually assessed for individuals that switched from actively "swimming" to an inactive state at the end of the treatment exposure ($n = 4$ chambers of 30–40 larvae per condition). We chose a rapid visual assay to measure putative larval behavioural changes given that (1) the bleaching-response was observed in the adults after only 12 h of deoxygenation, (2) we initially predicted that larvae would be more sensitive to deoxygenation stress, as F_v/F_m of larvae was significantly lowered in higher temperature treatments after 12 h in contrast to the relatively consistent F_v/F_m found in the corresponding adults (Putnam et al., 2010), and (3) we needed a rapid in vivo measurement to ensure sampling for transcriptomics was relatively similar to the adult study.

2.3 | RNA isolation and RNA-Seq

Total RNA was extracted using the Qiagen RNeasy mini kit modified for coral larvae (Supplementary protocol 1). RNA-Seq was done using the same procedures as described previously for adult *Acropora selago* (Alderdice et al., 2020). RNA quality was evaluated through NanoDrop ND-1000 and gel electrophoresis via the presence of intact 18S and 28S ribosomal RNA bands using an Agilent 2100 Bioanalyzer (Agilent Technologies). An Illumina TruSeq Stranded mRNA Library prep kit was used to (1) separate the mRNA from the total RNA via polyA+ selection and (2) generate 2 x 150 bp paired-end libraries for each sample with an average library size of 364 bp. Sequencing was performed on the Illumina HiSeq 4000 sequencer at the BioScience Core laboratory (BCL) at the King Abdullah University of Science and Technology (KAUST).

2.4 | Sequence data processing and analysis

The pipeline for processing the sequence data was the same as used previously for adult *Acropora* (Alderdice et al., 2020). Paired-end

reads were quality-assessed using FastQC v0.11.5. Trimmomatic v0.38 was applied to trim off the Illumina adaptors and low-quality regions. Each read was scanned using a 4-base window and cut if the quality Phred score dropped below 15 (SLIDINGWINDOW:4:15). Leading and trailing bases were removed if quality dropped below a score of 3 (LEADING:3 TRAILING:3). Trimmed reads with resulting lengths shorter than 50 bases were excluded (MINLEN:50). Each sample retained >90% of the paired-end read counts. Trimmed reads were then mapped using Bowtie2 v2.3.5.1 to the reference genomic gene set (n = 28188 genes) of *Acropora millepora* (available at: https://przeworskilab.com/wp-content/uploads/acropora-millepora-assembly.pdf). Mapping files were processed with SAMtools for the generation of a bam file and alignment quality check. Read counts were then calculated via eXpress-1.5.1-linux_x86_64 (Roberts & Pachter, 2013) for determination of differential gene expression (Data S1). Samples with <5 million mapped reads were not considered for downstream analysis (Table S3). Significantly differentially expressed genes (DEGs; Benjamin-Hochberg, FDR, adjusted p-value <.05) between treatment and control groups for each time point were determined using DESeq2 in R (Love et al., 2014). Heat maps of fragments per kilobase per million reads (FPKMs) were created using DESeq2 in R. KEGG mapper was used to assess presence of genes for different pathways based on the KEGG orthologue (KO) annotations from EggNOG (Data S2) with particular focus on the HIF-1 signalling pathway map (KEGG map04066). FPKM expression estimates were generated via eXpress. Transcripts annotated to the same gene name (e.g., *HSP90*) were considered combined, and FPKM expression estimates for the multiple transcripts were summed per sample. More specifically, transcripts annotated as HSP90 or corresponding isoforms were considered collectively in our analysis, given that previous studies showing HSP90 interactions with HIF used nonisoform specific HSP90 inhibitors (e.g., geldanamycin) or anti-hsp90 antibodies (Hur et al., 2002; Isaacs et al., 2002; Katschinski et al., 2004; Zhou et al., 2004). See Table S4 for KO annotations used for the genes of interest for FPKM analysis over time. Of note, the presence of key promoter regions, for example, HIF hypoxia responsive elements (HREs), in coral orthologs of target genes previously characterised in mammals still need to be confirmed to infer specific gene network signalling. Therefore, in this study we only describe those coral orthologs with similar dynamics in gene regulation. Also, as some of our results rely on EggNOG annotations, some genes warrant further confirmation with phylogenetic analyses to corroborate correct functional inference, for example, ESRRG (NR3 member; NR3B3). ESRRG was annotated based on *A. millepora* Emapper results from this study, however, in a previous study it was designated as a homologue of nuclear receptors (NR), NR3 (Grasso et al., 2001). In addition, a more recent study suggests cnidarians to possess a novel NR3 from clade E, whose ligand-binding capacities still remain to be determined (Khalturin et al., 2018). Data generated from eXpress can be found at the GitHub repository available at https://github.com/reefgenomics/coral_larvae_deoxygenation_RNASeq.

3 | RESULTS

3.1 | Phenotype and broad pattern transcriptional response

Based on a visual assessment, there were no apparent phenotypic differences with regard to coral planula behaviour between control and treatment samples at the end of the experiment, as all planulae maintained their "swimming" activity (n chambers of 30–40 larvae = 4 per condition), rather than switching to an inactive state under oxygen-reduced conditions (Table S5). To closer elucidate putative effects of deoxygenation-reoxygenation treatment on coral planula larvae, we evaluated the expression of 28 188 mapped genes at three time points (T1 = 0.5 h, T2 = 12 h of deoxygenation, T3 = 12 h of subsequent reoxygenation) using RNA-Seq. Samples were largely separated by condition at each time point; while T1 and T3 were clearly distinct, there was some overlap between hypoxia treated and control samples at T2 (Figure S2). Despite this similarity, gene ontology enrichment analysis showed genes predominantly annotated with terms associated with glycolysis at T2 when comparing conditions, highlighting a shift from aerobic to anaerobic respiration in response to deoxygenation stress (Table S6). The total number of differentially expressed genes (DEGs) between conditions remained relatively low for both T1 and T2 (0.36% vs. 1.36% DEGs out of all mapped genes, respectively: Table S7). In contrast to T1 and T2, samples subjected to deoxygenation-reoxygenation stress (T3) exhibited a large transcriptional difference between conditions with the number of DEGs increasing by at least 6-fold compared to T2 (9.01% vs. 1.36% DEGs out of all mapped genes, respectively: Table S7). To further elucidate how such overall patterns relate to the HRS, we assessed expression of key genes of the HIF gene system involved in both early developmental processes and in mitigating deoxygenation stress (Table 1) by analysing their gene expression (i.e., FPKM) between conditions for each time point (T1–3). Selected genes included those key to the coral HIF-HRS, previously described from the *Acropora selago* adults (Alderdice et al., 2020).

3.2 | Differential gene expression under deoxygenation associated with early development and O_2-dependent processes.

After 12 h of deoxygenation exposure (T2), no significant difference was apparent in *HIFα* gene expression between treatment and control samples. In contrast, *PHD2* expression was significantly higher in treatment samples by 3-fold (Figure 2a; FC_{log2} = 1.59 FDR < 0.05). Only HIF-target genes associated with promoting glycolysis, for example lactate dehydrogenase *(LDH)*, showed significantly greater expression in the treatment compared to control samples at T2 (Figure 2b,c; *LDHB* FC_{log2} = 1.17 FDR < 0.05). However, following a subsequent 12 h of reoxygenation (T3), the larvae demonstrated a particularly large transcriptional response when comparing deoxygenation treatment and control samples,

TABLE 1 Gene homologs selected for analysis associated with the HIF gene system. Full gene names were retrieved from EggNOG annotations or KEGG definitions

Abbrev.	Full name	Gene ID
HIFA/EPAS1	Hypoxia-inducible factor/PAS domain protein	Amillepora27208
PHD/EGLN1	Prolyl hydroxylase domain/Egl nine homologue	Amillepora27205
HSP90AB1	Heat shock protein 90	Amillepora15295
HSP90B1	Heat shock protein 90	Amillepora17004
ERR/ESRRG	Oestrogen-related receptor	Amillepora14094
KCNK17/18	Two-pore potassium channel (K2P)/potassium channel subfamily K member	Amillepora08546, Amillepora29632, Amillepora24154
LDHB	L-lactate dehydrogenase B	Amillepora06787
BCL-XL	B cell lymphoma 2 extra large	Amillepora02966, Amillepora16348
BCL2	B cell lymphoma 2	Amillepora17953, Amillepora04939
BNIP3	BCL2 adenovirus E1B 19 kDa interacting protein 3	Amillepora06222
GLUT4	Solute carrier facilitated glucose transporter 4	Amillepora19140
p27/CDKN1B	Cyclin-dependent kinase inhibitor	Amillepora05037
CD36	Scavenger receptor class B, member	Amillepora08250
POLRMT	RNA polymerase mitochondrial	Amillepora00763
NDUF	NADH dehydrogenase (ubiquinone)	Amillepora17040, Amillepora25997, Amillepora26610, Amillepora25738, Amillepora01316, Amillepora15596, Amillepora15599, Amillepora03045, Amillepora29583, Amillepora35968, Amillepora08911, Amillepora20899
CD73	5'-nucleotidase, ecto	Amillepora19917, Amillepora19916, Amillepora19918
PDK4	Pyruvate dehydrogenase kinase	Amillepora11832, Amillepora11830
HK	Hexokinase	Amillepora03684
PFK	Phosphohexokinase	Amillepora20896
PGK	Phosphoglycerate kinase	Amillepora25280
CD39	Ectonucleoside triphosphate diphosphohydrolase	Amillepora04344
Casp3	Apoptosis-related cysteine peptidase	Amillepora10135, Amillepora10141, Amillepora22145, Amillepora16944, Amillepora10138, Amillepora02123, Amillepora18963, Amillepora22147, Amillepora22143, Amillepora11061, Amillepora10139
GAPDH	Glyceraldehyde3phosphate dehydrogenase	Amillepora18960
ALDO	Fructose-bisphosphate aldolase	Amillepora22643
ENO1	Enolase	Amillepora33792
HES7	Hairy and enhancer of split 7	Amillepora17919, Amillepora17928, Amillepora17925
PAX6/7	Paired box	Amillepora02016, Amillepora02017, Amillepora02019
SHH	Sonic HedgeHog	Amillepora16922, Amillepora12689, Amillepora12705
HOX	Homeobox	Amillepora01204, Amillepora01205, Amillepora01206, Amillepora05682, Amillepora05719, Amillepora12806, Amillepora12809, Amillepora01207, Amillepora03675, Amillepora04832, Amillepora05592, Amillepora06229, Amillepora06234, Amillepora12303, Amillepora12422, Amillepora12423, Amillepora22199, Amillepora24224, Amillepora26577, Amillepora26578, Amillepora26579, Amillepora26580, Amillepora27446, Amillepora27447, Amillepora27448, Amillepora27449, Amillepora31667, Amillepora12237
NOTCH	Neurogenic locus notch homologue protein	Amillepora03193, Amillepora03811, Amillepora06476, Amillepora29162, Amillepora30592

with a 24-fold increase in the total number of differentially expressed genes from T1 to T3 (Figure 2b; Table S1). Interestingly, the differences in gene expression profiles at T3 corresponded with treatment samples possessing significantly lower expression of *HIFα*, *HSP90*, *ESRRG*, as well as HIF-targeted development regulators. The latter included Homeobox *HOX* genes, a family of transcription

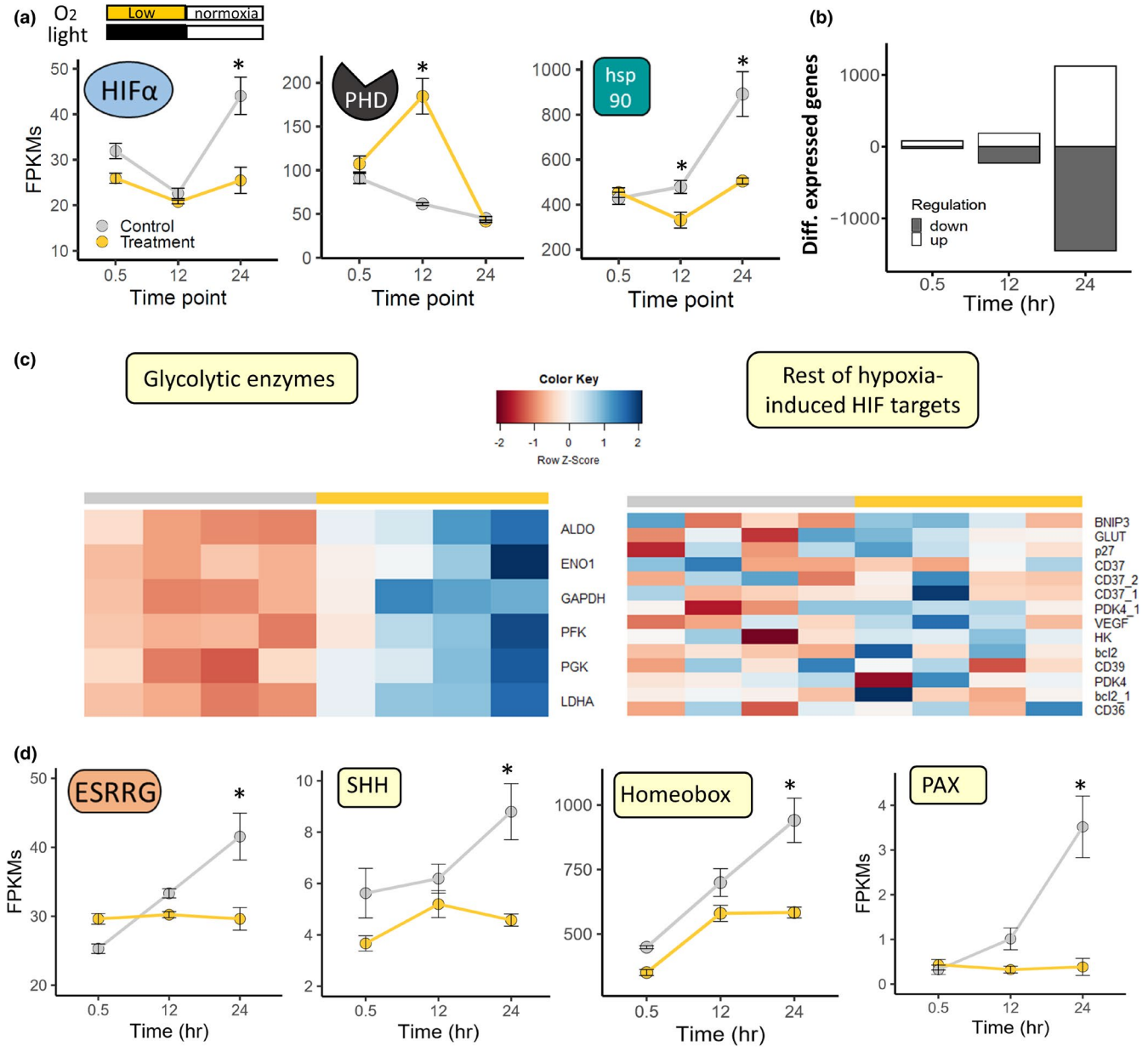


FIGURE 2 HIF-focused response of coral larvae to deoxygenation-reoxygenation exposure. (a) Fragments per kilobase per million mapped reads (FPKMs) of hypoxia-inducible factor alpha (*HIFα*), Prolyl hydroxylase domain (*PHD*), and heat shock protein 90 (*hsp90*) over 24 h, sampled at 0.5, 12 and 24 h for *Acropora selago* larvae under 12 h of night-time low oxygen and dark conditions followed by 12 h of oxygenated and daylight conditions. Yellow and white bars represent low and normal oxygen conditions. Black and white bars represent dark and light regimes following the night-day cycle. Error bars represent standard error. (b) Number of differentially expressed genes (DEGs) up (white) and down (grey) -regulated, comparing treatment (yellow) and control (grey) at each sampling point at 0.5, 12, and 24 h. (c) Heatmaps of FPKMs row z-score after 12 h of low oxygen exposure (T2) of HIF-targeted glycolytic enzymes and the remaining hypoxia-induced HIF targets listed in full Table S4. (d) FPKMs at each sampling time point when exposed to low oxygen (yellow) and normal oxygen (grey) levels for oestrogen-related receptors (*ESRRG*) and HIF-targeted development regulators (*SHH*, homeobox *HOX* and *PAX* genes). Symbols with error bars denote means ± standard error ($n = 4$). Asterisks represent differentially expressed genes by condition FDR < 0.05, $n = 4$

factors, that play a key role in the determination of the anterior-posterior axis during development (Pernice et al., 2006), suggesting disruption of vital early developmental processes when subjected to 12 h of deoxygenated seawater (Figure 2d; *HIFα*, *HSP90AB1*, *HSP90B1*, *ESRRG*, *SHH*, *HOXA3*, *PAX6* with FC_{log2} = −0.77, −0.86, −0.30, −0.46, −2.23, −0.27, −3.00 respectively at FDR < 0.05; Data S1). Moreover, two important genes of signalling development pathways, *Wnt* and *BMP4*, also followed similar gene expression dynamics, with a significantly lowered expression at T3 under treatment compared to control settings (Figure S3; *Wnt5*, *Wnt2B*, *Wnt4*, *Wnt10A*, *BMP4* with FC_{log2} = −2.71, −3.60, −1.89, −6.87, −0.36 respectively at FDR < 0.05), while the mesoderm-promoting growth factor Brachyury expressed significantly greater expression levels in treatment samples at T3 (Figure S3; FC_{log2} = 0.57 at FDR < 0.05).

To further understand the extent to which coral larvae were affected by exposure to deoxygenation, we next explored the expression patterns of genes encoding for O_2-associated receptors and genes involved in regulating mitochondrial activity, lipid metabolism, and O_2 -dependent epigenetic activity.

3.3 | O_2-associated receptors

All four O_2-associated membranal receptors we assessed – aquaporins (*AQP*), gamma-aminobutyric acid (*GABAr*), acetylcholine cholinergic (*CHRNr*), and melatonin (*MLTr*) – demonstrated greater gene expression in treatment samples compared to control after 12 h deoxygenation (T2; Figure 3) and exhibited a significantly greater expression level following return to normoxia for 12 h (T3; *AQP4*, *GABAr*, *CHRNr*, *MLTr* with $FC_{log2} = 0.43, 0.72, 1.30, 1.38$, respectively at FDR < 0.05; Data S1). These various receptors can function to increase oxygen transport (*AQP4*; Wang & Tajkhorshid, 2010; Zwiazek et al., 2017), signal to suppress oxygen-fueled respiration (*GABAr*; Wu et al., 2021), inhibit (*CHRNr*; Miao et al., 2013) and scavenge reactive oxygen species (*MLTr*; Yan et al., 2018; Buttar et al., 2020), and their increased expression highlight the impact on O_2 availability and ROS demands in coral larvae after 12 h of continuous deoxygenation exposure followed by reoxygenation. No significant difference in gene expression between treatment and control samples was observed for any of these receptors after 0.5 h of lowered oxygen (T1; Data S1), suggesting that coral larvae did not appear affected by shorter (or initial onset) deoxygenation exposure time. Expression of genes encoding for task-like two-pore domain (K2P) potassium channels were previously examined in the adult *Acropora* corals and were only upregulated at T1 in the more stress-tolerant species of *Acropora tenuis* (Alderdice et al., 2020), while a relatively low gene expression was found in *Acropora selago* larvae at each time point, with values less than half that found in the adult form (Figure S3).

3.4 | Mitochondrial activity

Expression of the gene *POLRMT*, key for mitochondrial biogenesis, was lower in treatment samples after both 0.5 and 12 h of deoxygenation exposure, indicating a reduced need to replace or generate more mitochondria (Figure 3). Expression of genes encoding NADH dehydrogenase (*NDUF*), a key enzyme catalysing mitochondrial complex I activity, was significantly reduced in treatment samples after 12 h of deoxygenation (*NDUFS7* $FC_{log2} = -0.30$, FDR < 0.05), an outcome also observed in adult *Acropora* under the same deoxygenation treatment (Alderdice et al., 2020). However, in the larvae *NDUF* expression was also significantly lower in treatment versus control samples after subsequent reoxygenation for 12 h (T3; $FC_{log2} = -0.40$, FDR < 0.05). Reduced *NDUF* expression in both cases may reflect the increase of HIF-targeted glycolytic enzymes expression, redirecting activity from the TCA cycle to the cytosol for anaerobic respiration (Figure S4). Hypoxia-induced mitophagy, here determined as the ratio of the expression of the pro-apoptosis gene *bnip3* (coding for the Bcl-2 19-kilodalton interacting protein) over the combined expression of the antiapoptosis genes *bcl2* and *bclxl* (B-cell lymphoma extra-large; Zhang & Ney, 2009; Pernice et al., 2011), was relatively similar in treatment versus control samples at both T2 and T3 (Figure S3).

3.5 | Lipid metabolism

Lipase (*LIPA*) gene expression was significantly higher at T2 in treatment samples compared to control (Figure 3; $FC_{log2} = 0.57$, FDR < 0.05), indicating the increased need for the larvae to access energy resources under a reduced oxygen environment. Phosphatidylserine decarboxylase (*PSD*), key to membranal phospholipid synthesis, demonstrated a significantly higher gene expression at T3 under treatment settings compared to control ($FC_{log2} = 1.81$, FDR < 0.05), indicating the need to enhance "structural lipid" production, probably as a means to repair degraded sites commonly experienced under oxidative stress.

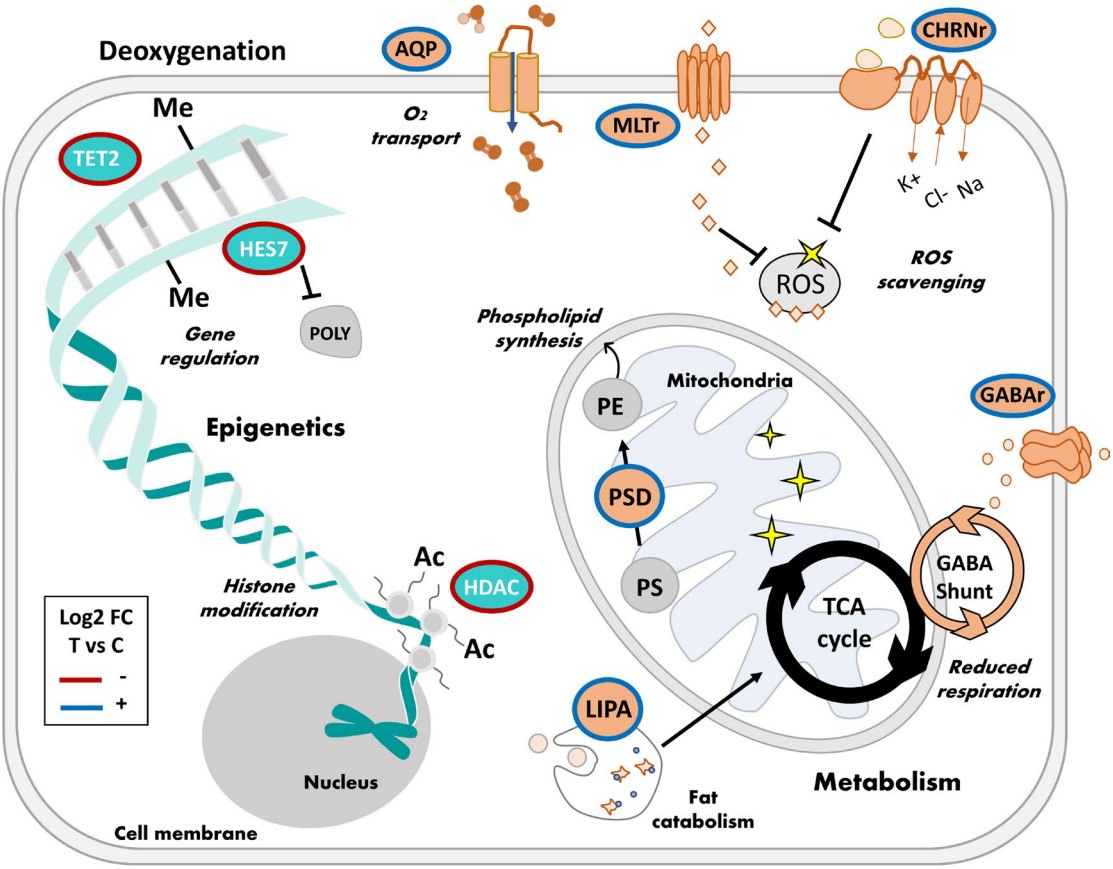
3.6 | Epigenetic activity

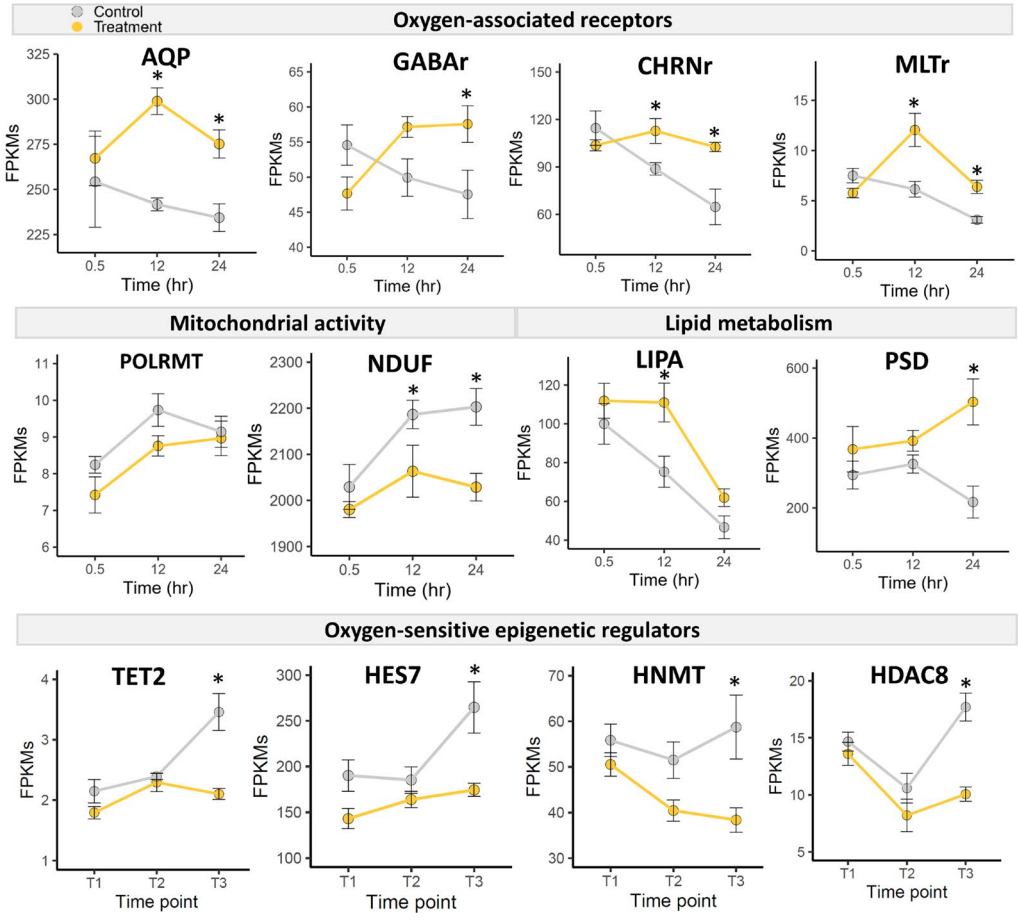
The O_2-sensitive epigenetic regulators Tet methylcytosine dioxygenase (*TET2*), *HES7*, histamine N-methyltransferase (*HNMT*), and histone deacetylase (*HDAC8*) all showed a significantly lower gene expression at T3, after deoxygenation-reoxygenation exposure in treatment samples, as compared to control (*TET2*, *HES7*, *HNMT*, *HDAC8* with $FC_{log2} = -0.69, -0.76, -0.98, -0.77$ at FDR < 0.05) indicating reduced levels of epigenetic activity under limited O_2 supply.

4 | DISCUSSION

Oxygen is a critical resource that governs a multitude of essential processes for coral and associated reef biota (Hughes et al., 2020; Jorissen & Nugues, 2020; Nelson & Altieri, 2019). Recent deoxygenation events on reefs have led to suggestions that hypoxia exposure is a major modulator of coral bleaching susceptibility (Hughes et al., 2020; Nelson & Altieri, 2019), which have been reinforced by

FIGURE 3 Non-HIF focused response of coral larvae to deoxygenation-reoxygenation exposure. Fragments per kilobase per million mapped reads (FPKMs) of O_2-associated receptors and O_2-sensitive epigenetic regulators as well as genes associated with mitochondrial activity and lipid metabolism (see list of full names in Table S7) over 24 h, sampled at 0.5, 12, and 24 h for *Acropora selago* larvae for treatment (yellow) and control (grey) with schematic of cellular context of selected genes. Symbols with error bars denote means ± standard error ($n = 4$). Asterisks represent differentially expressed genes by condition, FDR < 0.05, $n = 4$


Deoxygenation
AQP
O2 transport
MLTr
CHRNr
K+ Cl- Na
Me
TET2
HES7
Me
Gene regulation
POLY
ROS
ROS scavenging
Phospholipid synthesis
Mitochondria
PE
PSD
PS
GABAr
GABA Shunt
TCA cycle
Reduced respiration
Epigenetics
Ac
HDAC
Ac
Histone modification
Log2 FC T vs C
-
+
LIPA
Fat catabolism
Metabolism
Nucleus
Cell membrane


Control
Treatment
Oxygen-associated receptors
AQP
GABAr
CHRNr
MLTr
FPKMs
Time (hr)
Mitochondrial activity
Lipid metabolism
POLRMT
NDUF
LIPA
PSD
Oxygen-sensitive epigenetic regulators
TET2
HES7
HNMT
HDAC8
Time point
T1
T2
T3

experiments demonstrating how deoxygenation stress can rapidly drive adult forms of *Acropora* coral into a state of metabolic crisis that manifests as bleaching-induced mortality (Alderdice et al., 2020). Here, we explored how aposymbiotic coral planula larvae of the bleaching stress-susceptible coral *Acropora selago* respond to deoxygenation, with a particular focus on genes inferred to be associated with the HIF network, the hypoxia stress response, and O_2-dependent processes with regard to both: early developmental processes and mitigation of pathological effects due to limited oxygen supply.

We observed no apparent visual differences in planulae behaviour between control and treatment samples, as planulae maintained their swimming activity ($n = 4$ chambers of larvae per condition), rather than switching to an inactive state. This conforms to results from studies of other marine invertebrate larvae (from the phyla Chordata and Mollusca), where swimming behaviour was not affected when exposed to hypoxic O_2 levels (Kaufmann & Wieser, 1992; Mann & Rainer, 1990). Such behaviour could infer that those larvae possess a greater level of phenotypic tolerance to deoxygenation exposure compared to their adult life forms, which suffered bleaching-induced mortality under the same experimental treatment regime (Alderdice et al., 2020). However, quantitative phenotypic response data indicative of development succession, such as settlement success or rate, are needed for unequivocal validation. Despite the seeming indifference in larval phenotypes as indicated by their continued swimming behaviour, larvae that were exposed to deoxygenated conditions exhibited a pronounced shift in their transcriptional profiles. In the following, we examine the expression of genes in coral planula larvae between treatment and control after 0.5 and 12 h of deoxygenation exposure and after 12 h of subsequent reoxygenation.

Interestingly, when larvae were exposed to 12 h of deoxygenation stress (T2), the gene expression of the O_2-sensitive HIF subunit, *HIFα*, did not significantly differ between treatment and control conditions and exhibited much lower expression by at least 7-fold compared to those observed for adult corals under similar conditions (Alderdice et al., 2020). At the same time, *PHD2* expression that functions in promoting *HIFα* proteasomal degradation under normoxia (Rytkönen et al., 2011) was 3 times higher in larvae under treatment conditions at T2 (Figure 2). Similarly, *PHD2* was also differentially expressed in adult *A. selago* corals in response to 12 h of night-time deoxygenation (Alderdice et al., 2020). Overexpression of *PHD2* under hypoxic conditions has been reported to either induce *HIFα* proteasomal degradation during prolonged hypoxia or to prepare cells for rapid *HIFα* breakdown when reoxygenation occurs (D'Angelo et al., 2003; Philip et al., 2013). However, such a response was not observed in more bleaching-tolerant *A. tenuis* adult corals (Alderdice et al., 2020), indicating that *PHD2* overexpression under low O_2 may signal a level of susceptibility to deoxygenation stress whether in response to the lack of O_2 or increase in ROS. Figure 4 summarizes the expression of key genes associated with the HIF-HRS that may determine coral stress tolerance for different coral life stages. Of note, both the promoter regions and gene network interactions of these genes are inferred and will require confirmation by chromosome-scale coral genome assemblies, which are not yet available.

Among the studied HIF-target genes, only those encoding for glycolytic enzymes such as lactase hydrogenase (*LDH*) demonstrated differential expression between treatment and control samples at T2 (Figure 2b). Interestingly, a similar response was found in the adult forms of *A. selago* at T2 (Alderdice et al., 2020). Conversely under similar treatments, the more stress-tolerant adult species, *A. tenuis*, demonstrated a significantly greater expression of HIF-target genes such as *CD36* (promoting lipid uptake), as compared to adult *A. selago*. Thus, the low expression of HIF target genes in *A. selago* across both life stages indicates a lower inducibility of the HIF system in response to deoxygenation for this coral species (Alderdice et al., 2020).

Hypoxic microenvironmental cues are known to regulate proliferation and differentiation capabilities of cells during development in mammals (Abdollahi et al., 2011). Notably, such cells express the HIF gene network to signal for early developmental processes (see Figure 5) that require specific intra-tissue O_2 gradients (Hubbi & Semenza, 2015). In our study, following 12 h of deoxygenation and reoxygenation stress, a large number of differentially expressed genes (DEGs) exhibited lower expression in treatment samples (Figure 2b). Among the DEGs that were expressed lower in treatment samples, we found genes of interest annotated as (1) *HIFα*, (2) *HSP90*, a common molecular chaperone reported to stabilise HIFα proteins (Isaacs et al., 2002; Katschinski et al., 2004), (3) *ESRRG*, known to stimulate HIF-induced transcription during growth (Ao et al., 2008), and (4) development regulators (e.g., *SHH*, *HOX*, and *PAX genes*) reported as HIF targets in mammals (Bijlsma et al., 2009; Downes et al., 2018; Sinha et al., 2019) and found to be expressed in coral (Hemond et al., 2014). Such decreased gene expression for a number of cellular signalling genes suggests a potential interruption to key early development processes, including cell proliferation, after experiencing a reduced oxygen environment, whether signalled by HIF or not. Gene expression of *Wnt* and *BMP4* proteins involved in further conserved signalling pathways that stimulate early development (Ball et al., 2004) and have been previously reported in coral (Gutner-Hoch et al., 2017; Hemond et al., 2014), were also similarly affected under treatment conditions (Figure S3), emphasising the general disruption deoxygenation stress probably imposed on developmental processes in these larvae. However, the expression of *Brachyury*, encoding for a mesoderm-promoting growth factor (Ramírez-Bergeron et al., 2004), responded in a contrasting manner to these developmental signalling pathways and was upregulated under treatment and downregulated in controls (Figure S4). Together, these patterns therefore suggest key development signals may have become desynchronised under deoxygenation stress, which could give rise to developmental abnormalities commonly reported in heat and nutrient stressed *Acropora* coral embryos and larvae (Harrison & Ward, 2001; Humanes et al., 2016; Negri et al., 2007; Portune et al., 2010). Such disturbance to the growth of the larvae may be a temporary means to manage and reprioritise energy resources or may

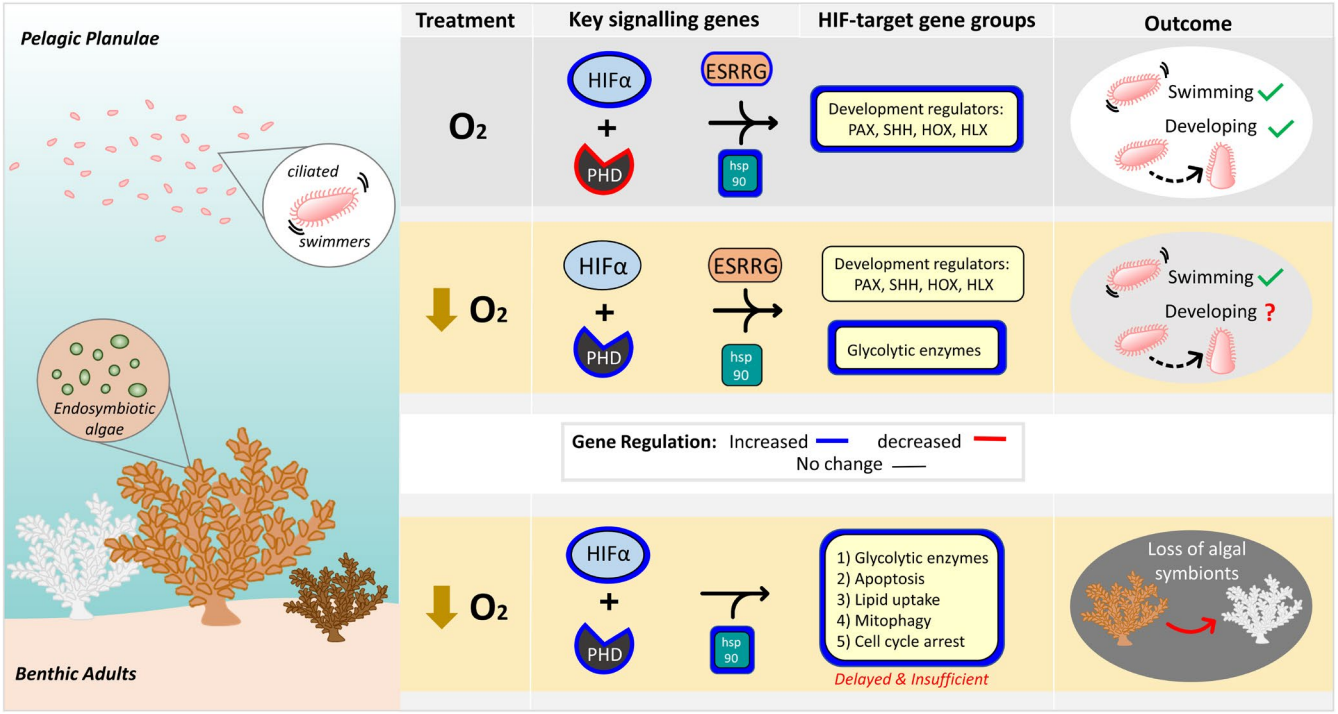
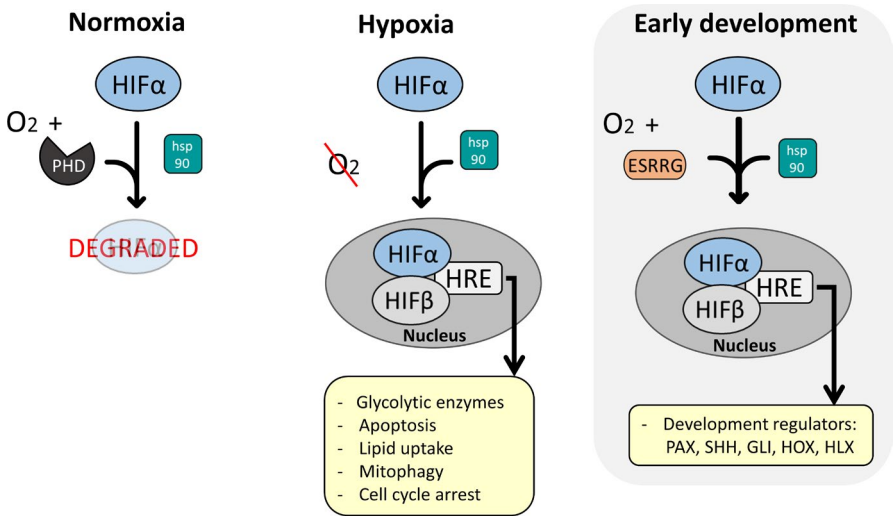


FIGURE 4 Key regulations in the inferred coral HIF gene system that could determine tolerance to hypoxia across different coral life history stages. Schematic comparison of *Acropora selago* life history stages, that is, pelagic mobile apo-symbiotic planulae and benthic immobile adults (adult data from Alderdice et al., 2020), with respect to their regulation of key HIF genes associated with early development and deoxygenation stress mitigation, plus the outcome of post-stress experiment. The colour gradient of coral from darker brown to white indicates a declining number of functional endosymbiotic algae present in coral affected by bleaching

FIGURE 5 The putative role of HIF in development of coral larvae. Schematic illustration based on mammal gene interactions of how the hypoxia-inducible factor (HIF) pathway is coordinated under normoxia (HIF degraded), hypoxia (activates transcription of hypoxia stress-mitigating target genes), and during early development stages in metazoans



point towards a negative latent effect on the success of the larvae. To evaluate the suggested differences in development progression, quantitative phenotypic data indicative of development succession would be required as well as the contextual gene expression provided by timepoint zero samples to unequivocally disentangle development progress from the impact of the deoxygenation treatment.

To gain more insight into the impact of deoxygenation exposure on larval energy stores and transcriptional signalling, we examined genes encoding for O_2-associated membranal receptors, mitochondrial activity (Alderdice et al., 2020; Lutz et al., 2015), lipid metabolising enzymes expressed in corals, and also O_2-sensitive epigenetic regulators. Genes encoding for the O_2-associated membranal receptors aquaporins (*AQP*), gamma-aminobutyric acid (*GABAr*), acetylcholine cholinergic (*CHRNr*), and melatonin (*MLTr*) demonstrated greater expression in treatment samples compared to control after 12 h (T2) but also after 12 h of reoxygenation (T3), highlighting how the O_2-associated stress signalling was still discernible, even once reoxygenated (Figure 3). GABAr, CHRNr, and

MTr are small, rapidly diffusible messenger molecules that play an important role in cell-cell communication in the neural and immune systems of animals (Iyer et al., 2004) and have been previously studied in relation to coral larval settlement and growth cues (Hemond et al., 2014; Mohamed et al., 2020; Siboni et al., 2012; Strader et al., 2018). NADH dehydrogenase (*NDUF*), an enzyme that regulates mitochondrial complex I activity and was previously linked to the host redox state in thermally stressed coral (Lutz et al., 2015), was significantly lower in treatment samples at T2 and T3 (Figure 3). This highlights the lower mitochondrial activity under deoxygenation-reoxygenation stress, where the shift from aerobic to anaerobic respiration is possibly promoted by HIF-target glycolytic enzymes to conserve O_2 supplies and reduce the mitochondrial ROS produced.

In alignment with previous evidence of rapid declines in lipid metabolism during coral larval stages to extend larval longevity (Graham et al., 2013), we found that gene expression of *LIPA*, encoding for a lipase enzyme that breaks down "energetic lipids" such as fats and triglycerides, was consistently lower in control samples at each time point with the lowest value at T3. Furthermore, *LIPA* expression was significantly higher at T2 in treatment samples (Figure 3). By comparision, the *PSD* gene encoding for Phosphatidylserine decarboxylase, an important enzyme in the synthesis of "structural lipids" such as phospholipids (Gsell et al., 2013), showed a significantly increased expression at T3 under treatment settings. Such apparent increases in "energetic" lipid metabolism and "structural" lipid synthesis in coral larvae under deoxygenation-reoxygenation stress indicate high energy demands and structural damage to cell membranes that would likely hinder developmental progress and competence of the larvae, as previously reported in thermal stress studies (Polato et al., 2013). Finally, the expression of genes encoding for the O_2-sensitive epigenetic regulators *TET2* (Solary et al., 2013), *HES7* (Zhou et al., 2008), *HNMT* (Waskiewicz et al., 1988), and *HDAC8* (Okazaki & Maltepe, 2006) were significantly lower in treatment samples at T3 compared to controls (Figure 3). This corroborates how O_2 gradients are key in tissues of developing animals, as cells acquire distinct O_2- dependent epigenetic landscapes that determine their function (Burr et al., 2018). Such genes could also be used as promising biomarkers of low-O_2 stress studies of HIF transcription (Watson et al., 2010) or genome-wide stress responses (Skiles et al., 2018), as we start to explore the role of epigenetics in influencing the capacity for different coral to adjust to climate change (Dimond & Roberts, 2016; Liew et al., 2020; Rodriguez-Casariego et al., 2018; Voolstra et al., 2021).

In summary, we found that swimming activity of *Acropora selago* planulae exposed to deoxygenation-reoxygenation stress did not cease. Based on our RNA-Seq analysis, gene expression of *HIFα* between treatment and control conditions after 12 h of deoxygenation (T2) was consistent. In contrast, *PHD2* involved in *HIFα*-degradation exhibited significantly higher gene expression in treatment samples. Not surprisingly, with such high *PHD2* levels only HIF-target genes associated with glycolysis were differentially expressed at T2. However, other genes associated with an inferred function in hypoxia and O_2-dependent processes were also differentially expressed, suggesting deoxygenation stress in the coral larvae. Interestingly, after subsequent reoxygenation (T3) we found that treated larvae showed a significantly lower expression of *HIFα*, *HSP90*, *ESRRG*, HIF-target genes, and classic development regulators. This may reflect either a temporary phase while larvae adjust to hypometabolism or may be indicative of negative latent developmental effects. An important next step will be to determine the presence of key promoter regions, for example HIF's HRE, in coral orthologs of target genes previously characterised in mammals and to examine such protein-protein interactions and validate key signalling gene networks (Ryu et al., 2011) under coral cellular hypoxia. However, this will require chromosome-scale assemblies, which are (largely) not available for coral genomes, although this may change in the not-too-distant future (Voolstra et al., 2021). Future work should include brooding coral species' larvae that already possess symbiotic algae, larvae from adults with known stress tolerance, longer stress exposure times to further determine the impact of ocean deoxygenation on settlement of coral planulae, and post-settlement monitoring of survival and growth rates, which will be important for coral larval restoration efforts.

ACKNOWLEDGEMENTS

We wish to particularly thank teams from James Cook University Cairns (led by Jamie Seymour, Katie Chartrand) and Southern Cross University (led by Peter Harrison, Dexter dela Cruz, Nadine Boulotte and Kerry Cameron) for their invaluable support in collecting corals used in this study (Vlasoff Reef, northern Great Barrier Reef) and subsequent holding in large-scale acclimation aquaria prior to experimentation, coral spawning and larval culture. All corals were collected under a Great Barrier Reef Marine Park Zoning Plan 2003 Part 5.4 Authorization (MPA18/002 "Coral larval restoration and Symbiodinium coculture collaborative project") to Peter Harrison. Also, immense thanks to the King Abdullah University of Science and Technology's (KAUST) Bioscience Core Laboratory (BCL) for assistance with Illumina sequencing. Funding for this work was supported by an Australian Research Council (ARC) Discovery Grant (DP180100074) to D.J.S., M.P., M.K. and C.R.V, and a Small Business Innovation Research 2018-2019 Project TF6.7.1 competitive grant to P.L.H., K.S. and D.J.S. Also, M.K. acknowledges support by the Gordon and Betty Moore Foundation (grant no. GBMF9206, https://doi.org/10.37807/GBMF9206) and C.R.V. acknowledges support by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) project numbers 433042944 and 458901010. Open access funding enabled and organized by Project DEAL through the University of Konstanz, Germany. Open access funding enabled and organized by ProjektDEAL.

CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

David J. Suggett, Mathieu Pernice, Christian R. Voolstra, Michael Kühl, David J. Hughes and Rachel Alderdice designed and conceived

the experiment; David J. Hughes and Rachel Alderdice collected the samples and conducted the experiment; Rachel Alderdice processed all samples; Anny Cárdenas generated the sequencing library preparations; Christian R. Voolstra, Anny Cárdenas and Rachel Alderdice analysed the RNA-Seq data; Rachel Alderdice conducted the statistics and generated figures; Rachel Alderdice and Christian R. Voolstra wrote the manuscript with input from all authors in their respective areas of expertise. All authors reviewed and approved the final manuscript.

OPEN RESEARCH BADGES

This article has earned an Open Data Badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available under NCBI BioProject PRJNA723188 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA723188) and GitHub repository available at https://github.com/reefgenomics/coral_larvae_deoxygenation_RNASeq.

DATA AVAILABILITY STATEMENT

Sequence data determined in this study have been made available under NCBI BioProject PRJNA723188: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA723188. Source data underlying figures and statistical analyses are provided in the Supporting Information. Scripts used in RNA-Seq data analysis pipeline can be found at the GitHub repository available at https://github.com/reefgenomics/coral_larvae_deoxygenation_RNASeq.

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How to cite this article: Alderdice, R., Pernice, M., Cárdenas, A., Hughes, D. J., Harrison, P. L., Boulotte, N., Chartrand, K., Kühl, M., Suggett, D. J., & Voolstra, C. R. (2022). Hypoxia as a physiological cue and a pathological stress for coral larvae. *Molecular Ecology*, 31, 571–587. https://doi.org/10.1111/mec.16259