Gobies inhabiting natural CO_2 seeps reveal acclimation strategies to long-term acidification

Sneha Suresh[1], Alice Mirasole[2], Timothy Ravasi[3,4], Salvatrice Vizzini[5,6], Celia Schunter[1,7*]

[1]Swire Institute of Marine Science, School of Biological Sciences, The University of Hong Kong, Pokfulam Road, Hong Kong SAR

[2]Department of Integrative Marine Ecology, Ischia Marine Centre, Stazione Zoologica Anton Dohrn, Punta San Pietro, 80077 Ischia, Naples, Italy

[3]Marine Climate Change Unit, Okinawa Institute of Science and Technology Graduate University, Onna-son, Japan

[4]Australian Research Council Centre of Excellence for Coral Reef Studies, James Cook University, Townsville, Australia

[5]University of Palermo, Department of Earth and Marine Sciences, via Archirafi 22, 90123 Palermo, Italy

[6]CoNISMa, National Inter-University Consortium for Marine Science, Piazzale Flaminio 9, 00196 Roma, Italy

[7]State Key Laboratory of Marine Pollution, City University of Hong Kong, Hong Kong, Hong Kong SAR, China

*Corresponding author

Correspondence: celiaschunter@gmail.com

Abstract

Changes in ocean chemistry resulting from increasing acidification have direct impacts on the physiology and behaviour of teleosts. Ocean acidification (OA) can also affect species behaviour, survival and fitness with repercussions at population, community and ecosystem levels. Volcanic CO_2 seeps offer a unique opportunity to study *in situ* both the direct and indirect effects of OA on fish and investigate their potential for acclimation. Here, we used the natural CO_2 seeps in Vulcano Island, Italy to study the effects of OA on the brain transcriptome of the anemone goby, a species with high population density in the CO_2 seeps. When compared to fish from environments with ambient pCO_2, gobies living in acidified waters showed differences in expression of transcripts involved in ion transport and pH homeostasis, cellular stress, and immune response indicating their capability to mitigate CO_2 induced oxidative stress and maintain physiological pH. We also found evidence of potential adaptive mechanisms to restore the functioning of GABA, whose activity can be affected by exposure to elevated CO_2 levels. There was also a conspicuous difference in expression of core circadian rhythm transcripts which could provide an adaptive advantage by increasing flexibility of physiological processes under acidified conditions. Additionally, an overall increase in metabolism in fish from the CO_2 seep indicates the ability to meet increased energetic costs associated with living in an acidified environment. Our results show potential molecular processes of acclimation to elevated pCO_2 in gobies enabling them to thrive in the acidified waters of Vulcano Island.

Keywords: Acclimation, shallow CO_2 vents, ocean acidification, transcriptomics, climate change, anemone goby

Introduction

Global change driven by anthropogenic activities is having profound effects on marine ecosystems with rising carbon dioxide (CO_2) levels being one of the most crucial problems (Doney et al., 2009, 2012; Hofmann et al., 2010; Kroeker et al., 2010). Oceans are major carbon sinks and have absorbed nearly 30% of anthropogenic CO_2 emissions since the 1980s resulting in seawater acidification (Bindoff et al., 2019). Elevated partial pressure of CO_2 (pCO_2) in seawater can result in hypercapnia in fish and other organisms. However, fish are able to successfully compensate for reduction in extracellular pH by uptake and/or retention of plasma HCO_3^- or net excretion of H^+ in exchange for counterions such as Cl^-, Na^+ and/or K^+ (Brauner et al., 2019; Ishimatsu et al., 2005). While this ability of fish to effectively regulate and maintain pH within physiological limits is advantageous, it leads to increased energy requirements and altered ionic composition resulting in various physiological and behavioural effects (Heuer et al., 2019; Heuer & Grosell, 2014). Exposure to elevated CO_2 has shown to impact the physiology, growth rate, development, survival, and reproduction in various species of fish (Esbaugh, 2017; Rachael M. Heuer & Grosell, 2014). Additionally, ocean acidification is known to cause cognitive impairment and altered behaviour in fish (Domenici et al., 2012; Ferrari et al., 2012; S. L. Hamilton et al., 2017; T. J. Hamilton et al., 2014; Munday et al., 2010; Munday, Pratchett, et al., 2013; Radford et al., 2021; Simpson et al., 2011) which in turn can affect species interactions ultimately impacting ecosystem functioning (Fabry et al., 2008; Kroeker et al., 2010). The CO_2 induced disruption of innate behaviour of fish has direct effects on survival and fitness which can have far-reaching consequences on sustainability of entire populations (Ferrari et al., 2011; Munday et al., 2010). It has been proposed that the behavioural effects of CO_2 on fish is the result of changes in ionic composition due to pH buffering processes, specifically increase in plasma HCO_3^- and a corresponding decrease in Cl^- concentrations which affects the functioning of gamma-Aminobutyric acid (GABA), the major

inhibitory neurotransmitter in the brain (Heuer et al., 2016; Nilsson et al., 2012; Schunter et al., 2019). Functional genomic studies on fish have reported changes in the expression of genes involved in the GABAergic signalling pathway and circadian rhythm in response to elevated CO_2 suggesting that CO_2 affects neuronal activity which could in turn affect behaviour (Kang et al., 2022; Lai et al., 2016; Schunter et al., 2016, 2018).

The majority of our knowledge about these impacts of OA on teleosts, however, comes from short-term laboratory-based studies and mostly only account for short-term effects of OA. Accurate assessment of the future biological impacts of OA on species and populations requires consideration of their potential for acclimation and adaptive evolution (Sunday et al., 2014). There exists substantial variability in sensitivity to elevated CO_2 both within and across species which can result in selection of CO_2 tolerant individuals or species in nature (Ferrari et al., 2011; Munday, McCormick, et al., 2013). In fact, studies have reported heritability of behavioural response to high CO_2 (Welch & Munday, 2017) and intergenerational acclimation of species to elevated CO_2 (Allan et al., 2014; Munday, 2014; Schunter et al., 2016, 2018; Stiasny et al., 2018; Strader et al., 2020; Tsang et al., 2020) which could potentially result in adaptation to ocean acidification over multiple generations. Additionally, other environmental factors such as nutrient gradients, species composition, habitat modifications, and predation risk can interact with CO_2 to affect species responses to OA (Mirasole et al., 2019; Munday et al., 2014; Nagelkerken et al., 2015; Zunino et al., 2019). Volcanic CO_2 seeps in the ocean, where emission of CO_2-rich fluids locally reduce pH, can be used as natural analogues to study the effects of long-term exposure of fish to elevated CO_2 in their natural environment. CO_2 seeps provide a unique opportunity for integrating the direct and indirect effects of OA on fish physiology and behaviour under natural conditions and estimating their adaptive potential. Increased primary productivity at CO_2 seeps for instance may help certain fish species to meet the increased energy demands of living in a high CO_2 environment (Connell et al., 2013). Shifts

in habitat forming species, trophic diversity and fish assemblages at CO_2 seeps have been reported which could obscure some of the negative effects of OA on fish behaviour (Fabricius et al., 2011; Hall-Spencer et al., 2008; Mirasole et al., 2019; Nagelkerken et al., 2015; Vizzini et al., 2017). For example, gobies from the CO_2 seeps in Vulcano Island, Italy living in more open habitats showed reduced predator escape behaviour, however, the same was not observed in gobies living in more sheltered sites indicating the influence of habitat on species response to OA (Nagelkerken et al., 2015). Predation risk is another factor that can antagonistically interact with CO_2 to influence species behaviour. For example, the predator recognition ability of the temperate wrasse (*Symphodus ocellatus*) from the CO_2 seeps in Vulcano Island was unaffected by elevated CO_2 possibly due to similarly high predator abundance at vent sites compared to sites with ambient CO_2 levels (Cattano et al., 2017) however, fish from the tropical CO_2 seeps in Papua New Guinea, where the CO_2 vents have reduced predator abundance, showed impairment in predator recognition ability (Munday et al., 2014). Additionally, benthic species such as *S. ocellatus* where multiple generations are exposed to the same elevated CO_2 levels, could develop mechanisms of resilience to OA that is passed on across generations enabling them to compensate the negative effects of elevated pCO_2 (Cattano et al., 2016). Perturbations in reproductive behaviour were not observed in *S. ocellatus* living in the natural CO_2 seep sites in Vulcano Island and although elevated CO_2 did negatively affect spawning behaviour of dominant males, reproductive success did not decrease (Milazzo et al., 2016). Long-term exposure to elevated CO_2 at the seeps may enable fish to develop tolerance through processes enabling physiological and behavioural compensation. Evidence from studies using transcriptomics to determine gene expression patterns in the brain and gonads of fish from natural CO_2 seeps revealed plasticity and adaptive selection to elevated CO_2 (Kang et al., 2022; Petit-Marty et al., 2021). These findings suggest that species that are continuously exposed to

elevated CO_2 levels can develop mechanisms to mitigate the effects of CO_2 exposure and have the potential to adapt to acidified environments.

While CO_2 alone affects various aspects of fish physiology and behaviour, these effects are not independent of the environment which in turn is also affected by changes in CO_2 concentrations. For example, net resource enrichment at CO_2 seeps in the Mediterranean seems to benefit crypto- and necto-benthic species such as gobies by providing sufficient food to meet the increased energy demands of maintaining pH and ion homeostasis (Mirasole et al., 2019; Nagelkerken et al., 2015). Even though potentially negative physiological effects such as changes in otolith morphology and skeletal composition have been reported in *Gobius* from the natural CO_2 seeps at Vulcano Island (Mirasole et al., 2017, 2021), they have higher population densities at the seeps possibly facilitated by CO_2 induced increase in resource availability (Nagelkerken et al., 2015). This indicates that the gobies may have developed acclimation strategies to acidified environments. To study the molecular pathways underlying acclimation to elevated CO_2 in wild populations of gobies, while also accounting for the indirect effects of OA, here we sequenced and analysed the brain transcriptome of the anemone goby, *Gobius incognitus* from the natural CO_2 seeps in Vulcano Island, Italy. By examining brain gene expression patterns we aim to identify molecular signatures of acclimation that enable this species to mitigate negative effects of elevated CO_2 and thrive in the acidified waters of Vulcano Island.

Methods

Study area and sample collection

Gobius incognitus samples were collected on 30th September 2018 at a depth of 1-2 meters with small hand nets on SCUBA from two locations in Levante Bay, Vulcano Island (Italy): a low pH site (38.419 ºN, 14.961 ºE; pH 7.80 ± 0.09) and a control site located around

600 m from the low pH site (38.420 ºN, 14.965 ºE; pH 8.19 ± 0.03). The control site was similar to the low pH site in terms of orientation (south-east), depth and habitat (sandy with beds of *Cymodocea nodosa* seagrass). A total of eight individuals were sampled at each site at the same time of day (between 11:00 am and 12:00 pm). The total length of all fish was measured in the field, immediately after which they were euthanized on ice and the brain tissues were dissected, preserved in RNAlater, and stored at -80ºC until further processing. The age of all sixteen individuals was estimated using the Von Bertalanffy function (Supplementary Table S1).

RNA extraction and sequencing

Frozen whole brains were homogenized in RLT-lysis buffer (Qiagen) for 30s at 30Hz/s using Qiagen TissueLyzer II with 4-5 single-use silicon beads per sample and total RNA was extracted using the Qiagen RNeasy Micro Kit. On-column DNase digestion was performed using RNase-Free DNase1 (Qiagen) following the manufacturer's instructions, to remove any DNA contamination. RNA quality was determined using Qubit and Agilent 2100 Bioanalyzer following which the samples (eight from control and eight from the low pH site) were sequenced on an Illumina NovaSeq 6000 to get 151 bp, paired-end reads at the Centre for PanorOmics Sciences, The University of Hong Kong.

Sequence processing and primary *de novo* transcriptome assembly

The quality of all the sequencing reads was determined using FastQC v0.11.5 (Andrews, 2010). To ensure high quality of all reads, we trimmed adapters and sequences with an average Phred-scaled quality score below 30 using fastp v0.20.0 (Chen et al., 2018) with a 4-base sliding window approach. Sequences less than 40 bp in length were removed (Supplementary Table S2). The trimmed sequences were further filtered to remove potential contaminants, ambiguous bases, and rRNA sequences. Potential contaminant sequences were removed using kraken v2.0.8-beta (Wood & Salzberg, 2014) with a confidence score of 0.3 using the bacterial,

fungal, and viral databases from NCBI RefSeq as reference. For sequences containing ambiguous base calls (Ns), the longest sub-sequence of at least 40 bp without any ambiguous bases was extracted and retained if it was at least half the original sequence length and had an average Phred-scaled quality score above 30 using the 'filter_illumina' script from DRAP (Cabau et al., 2017). Reads originating from ribosomal RNA (rRNA) were identified and removed by mapping the sequences to the SSUParc (SILVA_138_SSUParc_tax_silva.full_metadata.gz) and LSUParc datasets (SILVA_132_LSUParc.full_metadata.gz) from SILVA (Quast et al., 2013) using Bowtie2 v2.4.1 (Langmead & Salzberg, 2012) in the very sensitive, local mode. The adapter free, filtered, quality-trimmed, and decontaminated reads from all 16 samples were then merged and assembled *de novo* using Trinity v2.8.4 (Grabherr et al., 2013) within DRAP v1.92 (parameters: -c '-x'; --no-trim; -m bwa; --no-rate) using the pooled-assembly strategy.

De novo **transcriptome assembly optimization**

Candidate coding regions (open reading frames (ORFs)) within the transcript contigs assembled by DRAP were identified using TransDecoder v5.5.0 (Haas et al., 2013) and only contigs with ORFs of at least 100 amino acids long were retained. These contigs were then blasted against the UniProtKB/Swiss-Prot Release 2021_01 database (Apweiler, 2004) using the BLASTX algorithm (Blast+ v2.6.0) (Altschul et al., 1990) with an e-value cut-off 1e-05 and retaining only one blast hit per contig (--max_target_seqs = 1). For each assembled contig only the best ORF was retained prioritized first based on blast homology and then based on ORF length (--retain_blastp_hits; --single_best_only). The quality of the selected contigs was further assessed using the runAssesment module within DRAP v1.92 and putative chimeric contigs were removed. The high-quality RNA-seq reads were then re-mapped to the filtered transcriptome assembly using Bowtie2 v2.4.1 in the sensitive mode and disallowing any discordant and unpaired alignments, but without any upper limit on the number of alignments.

To identify and cluster individually assembled contigs belonging to the same gene, the alignment results were used by Corset v1.09 (Davidson & Oshlack, 2014) to hierarchically cluster contigs based on the proportion of multi-mapped reads and the expression patterns. For each of the Corset generated clusters the longest transcript sequence was extracted using the fetchClusterSeqs.py script from Corset-tools (https://github.com/Adamtaranto/Corset-tools) and those clusters with no aligned reads were removed from the *de novo* transcriptome assembly.

To further validate the transcriptome assembly, blast search (TBLASTX, e-value = 1e-05, max_target_seqs = 1) was performed against the complete cDNA sequence of *Neogobius melanostomus* (round goby) obtained from Ensembl (release-104) (Howe et al., 2021), the phylogenetically closest species with a sequenced genome to *Gobius incognitus* (Thacker & Roje, 2011). To further reduce transcript redundancy and identify potential alternative transcripts that failed to cluster in the previous steps, the blast results were filtered to retain only the longest transcript for each blast hit to get the final *de novo* transcriptome assembly. Transcripts with no blast hits to *Neogobius melanostomus* were also retained in the final assembly as these could potentially be transcripts that are specific to our target species, *Gobius incognitus*.

Transcriptome annotation and evaluation of assembly completeness

The *de novo* assembled transcriptome was compared against the UniProtKB/Swiss-Prot Release 2021_01 database using BLASTX (e-value = 1e-05 and HSP length cut-off = 33) followed by Gene Ontology (GO) mapping and annotation within OmicsBox v1.4.11 (Gö Tz et al., 2008). InterProScan was used to identify the protein domains within the transcript sequences and the InterProScan GOs were merged with the GO annotations to get the annotated

transcriptome. For contigs that did not blast to the Swiss-Prot database, the Ensembl gene ID obtained by blasting against *Neogobius melanostomus* was retained.

The contiguity of the transcriptome assembly was determined by calculating various metrics using rnaQUAST v2.1.0 (Bushmanova et al., 2016), 'stats.sh' script from BBMap v38.87 (Bushnell, 2014) and 'contig_ExN50_statistic.pl' from Trinity accessory scripts (https://github.com/trinityrnaseq/trinityrnaseq/tree/master/util/misc). To incorporate the transcript expression levels in evaluating the contiguity of the *de novo* assembly, the ExN50 (expression-informed N50) metric was determined. ExN50 (expression-informed N50) represents the N50 value for the most highly expressed transcripts representing x% of the total normalized expression data and is more appropriate for transcriptome assemblies than the conventional N50 statistic (Galachyants et al., 2019). A peak of the ExN50 value at higher x percentiles indicates the effectiveness of the *de novo* assembly pipeline to assemble longer transcripts (Sahraeian et al., 2017). The expression levels of the transcripts were quantified using RSEM v1.3.1 (Li & Dewey, 2014) and the ExN50 value was calculated across different expression percentiles using the TMM normalized expression values. The level of completeness of the transcriptome assembly was determined using BUSCO v4.1.3 (Manni et al., 2021) using the Actinopterygii_odb10 and Eukaryota_odb10 sets of Benchmarking Universal Single-Copy Orthologs (BUSCO).

Differential expression analysis

The high-quality, filtered RNA-seq reads were re-mapped to the final transcriptome assembly using Bowtie2 v2.4.1 in the sensitive mode, disallowing any discordant and unpaired alignments and using additional parameters (--dpad 0 --gbar 99999999 --mp 1,1 --np 1 --score-min L,0,-0.1) to ensure the resulting alignment files are compatible with RSEM, which was used to quantify the transcript expression levels. Principal component analysis (PCA) was

performed to determine the overall transcript expression patterns in R v4.1.1 (R Core Team, 2021) following which differential expression analysis was done using DESeq2 v1.32.0 (Love et al., 2014). Transcripts that were not expressed in any of the 16 individuals were excluded from the DE analysis. The transcripts were considered to be significantly differentially expressed between the control and CO_2 seep sites if the FDR corrected p-value (padj) was less than 0.05, absolute log2 fold change (abs log2FC) was greater than 0.3 and baseMean was greater than 10. Enrichment of GO functional categories among the significant DE genes were determined using Fisher's Exact Test in OmicsBox v1.4.11 (OmicsBox – Bioinformatics Made Easy, BioBam Bioinformatics, March 3, 2019) with an FDR threshold of 0.05, using the option "reduce to most specific" to reduce the redundancy in the enriched GO categories identified.

Results

De novo transcriptome assembly

Out of the 33 ± 2.8 million raw read pairs obtained from the 16 RNA libraries sequenced 26 ± 2.5 million paired reads were retained after stringent filtering processes which was then used to construct the *de novo* transcriptome assembly (Supplementary Table S2). The primary *de novo* transcriptome assembly consists of 633,517 contigs. After retaining only the single best ORF per contig, removing chimeric sequences, clustering contigs and removing redundant or falsely assembled transcripts, the final transcriptome assembly has 43,349 transcripts (Supplementary Table S3). 56.4% (24,454) of these transcripts had blast hits to known proteins from the UniProtKB/Swiss-Prot database, whereas an additional 21% (9,362) had blast hits to the cDNA sequence of *Neogobius melanostomus*, leading to 78% of transcripts in the final *de novo* assembly with a gene ontology annotation (Supplementary Table S3 and S4).

Various metrics were computed to determine the contiguity of the assembly. The assembly had an N50 of 3,545, L50 of 7,998, and 26,341 (61%) contigs were over 1 Kbp in

length (Supplementary Figure S1**;** Supplementary Table S3). Furthermore, there was a high coverage of the longer transcripts indicated by a peak of ExN50 value at E86 (Supplementary Table S5**,** Supplementary Figure S2). On average 72% of the RNA-Seq reads mapped back to the assembled transcriptome (Supplementary Table S6) indicating sufficiently high quality of the *de novo* transcriptome. Analysis of transcriptome completeness using BUSCO revealed high representation of both the Actinopterygian core genes and the Eukaryota core genes. Specifically, 76.4% and 91.4% of Actinopterygii core genes and Eukaryota core genes respectively, were recovered completely. An additional 7.7% of Actinopterygii core genes and 7.1% of Eukaryota core genes were recovered partially (Supplementary Figure S3).

Differential transcript expression

Fish from CO_2 seep showed more transcriptional variation among individuals compared to fish from the control site (Figure 1). Among the 43,301 expressed transcripts across all 16 samples, 996 transcripts were significantly differentially expressed (DE) between the control and CO_2 seep sites (padj < 0.05, abs log2FC > 0.3, baseMean > 10; Supplementary Table S7). A total of 434 GO terms were enriched among the DE transcripts (Supplementary Table S8) which were categorized into broader functional groups such as acid-base and ion homeostasis, neural activity and related effects, circadian rhythm, metabolism, cellular stress response, and immune response (Supplementary Table S9). 68% of DE transcripts (679) were expressed at higher levels in fish from the CO_2 seep site compared to the control site (Supplementary Figure S4).

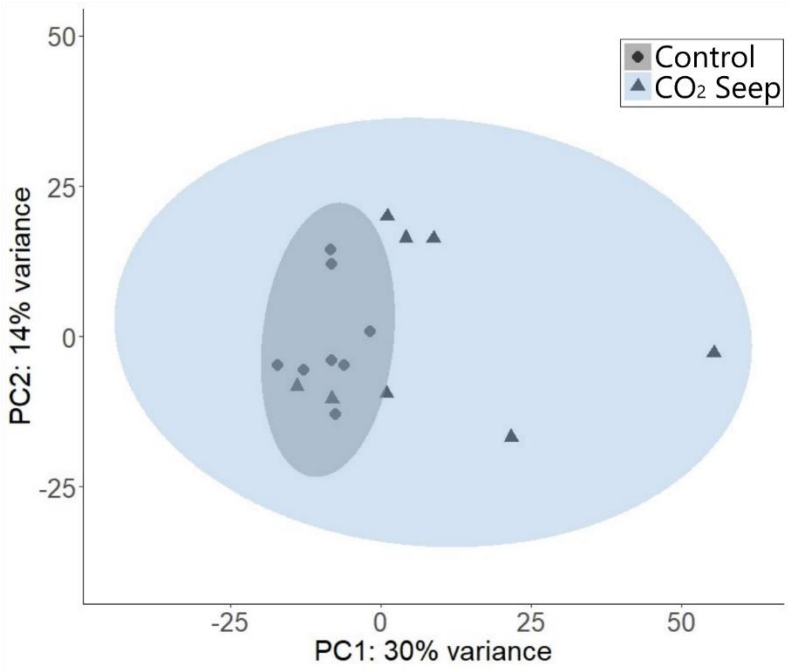

Figure 1: Overall transcript expression pattern across the 16 samples from the natural CO_2 seep and a control site at Vulcano Island. The PCA was performed using the regularized log transformed (rlog) counts of all expressed transcripts.

The five most significant DE transcripts were all associated with the circadian rhythm. These include two core circadian genes *PER3* and *NR1D2*, two nuclear hormone receptor genes involved in transcriptional regulation of core circadian genes *RORA* and *RORB*, and a transcriptional regulator known to regulate circadian rhythm genes *NFIL3* (Supplementary Figure S4). In fact, all core clock transcripts were DE between the control and CO_2 seep site; specifically, there was an overall downregulation of repressors of the circadian rhythm and upregulation of the activators. In addition to *NFIL3*, two more transcription factors (*CREB1*, *MTA1*), a splicing factor (*SFPQ*), and a histone demethylase (*KDM5A*) involved in the regulation of circadian rhythm genes were also found to be DE. Furthermore, *NOCT* a circadian deadenylase that functions as a post-transcriptional modulator in circadian control of metabolic genes was upregulated in individuals from the CO_2 seep (Figure 2; Supplementary Table S9).

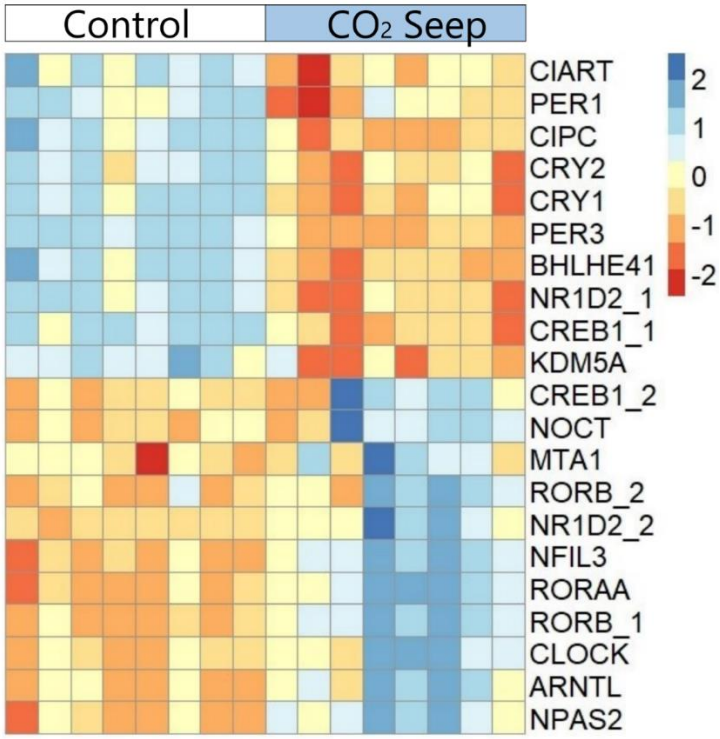

Figure 2: Relative expression levels of transcripts associated with the circadian rhythm of fish from control and CO_2 seep sites. The row z-score is calculated from the log2 transformed transcripts per million (TPM) values for each transcript.

Transcripts encoding various ion channels and transporters involved in the transport of potassium, sodium, and chloride were found to be differentially expressed between fish from the CO_2 seep and control sites (Figure 3a; Supplementary Table S9). Furthermore, transcripts encoding the Na^+/K^+ transporting ATPase (*ATP1A1*) and carbonic anhydrase 10 (*CA10*), which play critical roles in ion-regulation and maintaining pH homeostasis, were upregulated in fish from the CO_2 seep site (Figure 3a). Additionally, DE of various transcripts involved in actin cytoskeleton organisation (Supplementary Table S9) could indirectly affect membrane transport as the cytoskeleton closely interacts with the plasma membrane.

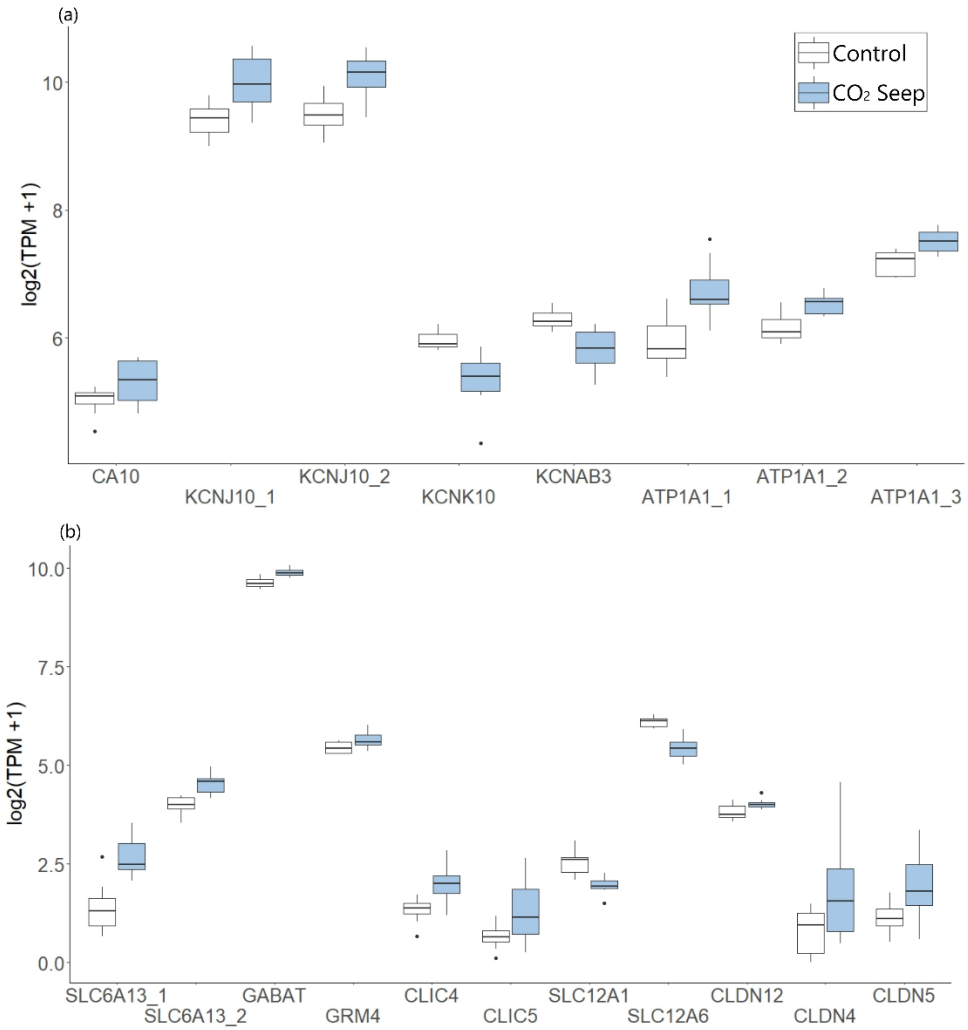

Figure 3: Expression levels of transcripts involved in (a) acid-base and ion homeostasis neurological functioning and (b) neurological functioning in fish from control sites and the CO_2 seep site. The relative expression level of the transcripts is calculated from the log2 transformed transcript per million (TPM) values. The lines indicate the lower and upper quartile corresponding to the 25th and 75th percentiles and the points indicate the data falling outside the lower quartile and upper quartile.

Changes in ionic composition across neuronal membranes can affect the activity of neurotransmitters such as GABA and glutamate and we found transcripts involved in degradation of GABA (*SLC6A13* and *GABAT*) and a transcript encoding the metabotropic glutamate receptor 4 (*GRM4*) to be differentially expressed in our study (Figure 3b; Supplementary Table S9). Additionally, transcripts encoding chloride transporters and claudins, whose expression is affected by neural activity, were DE (Figure 3b; Supplementary Table S9).

Metabolism was another function that was differentially regulated between fish from control and CO_2 seep sites. Transcripts involved in key metabolic processes that result in increased ATP production such as glycolysis, gluconeogenesis, TCA cycle, mitochondrial beta-oxidation, mitochondrial respiratory chain, lipid and amino acid metabolism were expressed at higher levels in gobies from the CO_2 seep site (Figure 4a). Interestingly, transcripts involved in fatty acid synthesis, an ATP consuming process were downregulated in individuals from the CO_2 seep (Figure 4a). Additionally, regulators of metabolic enzymes and transcripts involved in transport of glucose and other metabolites and a transcript encoding an ADP/ATP translocase (*SLC25A5*) were DE (Figure 4b; Supplementary Table S9).

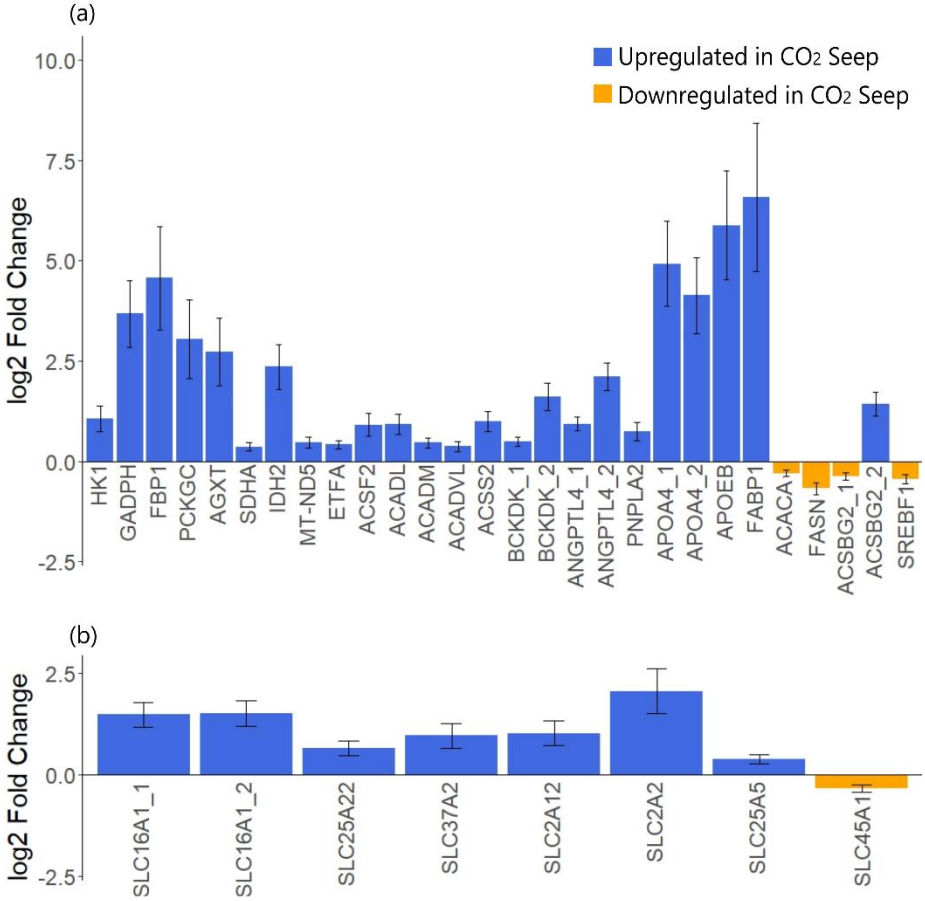

Figure 4: Differential expression levels (log2 fold change (log2FC)) of transcripts involved in (a) metabolism and (b) transport of metabolites between fish from control and CO_2 seep sites. The up-regulated transcripts in the CO_2 seep are in blue, and the downregulated transcripts are in orange. The error bars represent the standard error estimate for the calculated log2FC.

Transcripts known to play a role in the production of reactive oxygen species (ROS) such as those encoding angiotensin, epidermal growth factor, platelet derived growth factor receptor, members of the tumor necrosis factor (TNF) superfamily, and transcripts involved in phosphatidylinositol 3-kinase (PI3K) activity and signalling were DE. Additionally, various members of the Ras superfamily of small GTPases were DE. We also found transcripts associated with redox-sensitive signalling pathways such as the NF-*k*B and MAPK signalling to be DE. Transcripts involved in DNA damage repair, unfolded protein response and ubiquitination, molecular chaperones including heat shock proteins (HSPs), cell cycle control and apoptosis were also DE. Additionally, the expression levels of transcripts involved in cellular detoxification and redox regulation such as glutathione transferase, glutathione peroxidase, heme oxygenase, isocitrate dehydrogenase, and succinate dehydrogenase were higher in fish from the CO_2 seep (Figure 5; Supplementary Table S9). There was also an overall increase in the expression of transcripts associated with immune response and immune system function, and cellular response to hypoxia in individuals from the CO_2 seep site (Supplementary Table S9). Of particular interest was the *galactose-specific lectin nattectin*, a C-type lectin which is involved in innate immune responses as it had the highest fold-change in expression among all DE transcripts.

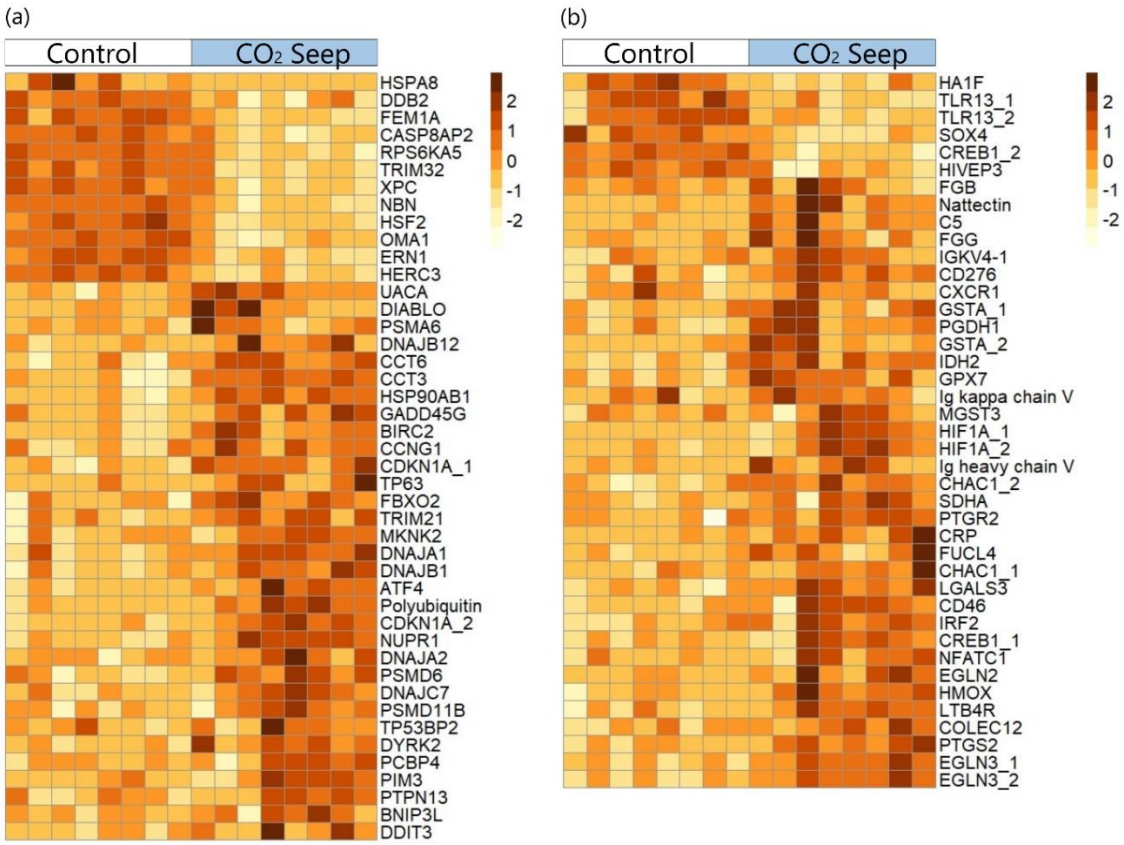

Figure 5: Relative expression patterns of transcripts associated with (a) DNA damage response, unfolded protein response, apoptosis and the HSP family of chaperons and (b) detoxification and redox regulation and immune response in fish from control and CO_2 seep sites. Each row represents the relative expression level of one transcript calculated in terms of the z-score from the log2 transformed Transcripts per million (TPM) values.

Discussion

Natural CO_2 seeps provide an excellent opportunity to study the effects of long-term exposure of fish to elevated CO_2 in their natural environment. Here, we investigated changes in the brain transcriptome of the anemone goby from a natural CO_2 seep in Vulcano Island to identify molecular processes that enable this species to thrive in these acidified waters. 2.3% of the brain transcriptome involved in key functions such as acid-base and ion homeostasis, neurological function, circadian rhythm, metabolism, cellular stress response, and immune response were differentially expressed (DE) between fish from the CO_2 seep and control sites. The majority of DE transcripts exhibited elevated expression in fish from the CO_2 seep similar

to what has been observed in gonads of the common triplefin from the CO_2 seep in New Zealand, another species known to have increased population size in acidified environments (Petit-Marty et al., 2021). The gobies seem to be able to elicit acclimation responses to elevated CO_2 by differentially regulating transcripts associated with crucial functions which potentially enables them to maintain cellular homeostasis and thrive in the acidified waters of Vulcano Island.

Exposure to elevated CO_2 can induce oxidative stress in fish which can trigger cell damage and apoptosis (Pizzino et al., 2017; Strader et al., 2020). Transcripts known to induce an increase in the intracellular concentration of reactive oxygen species (ROS), including various members of the Ras superfamily of small GTPases, were differentially expressed (DE) indicating that gobies from the seep are under oxidative stress (Poli et al., 2012). Additionally, we also observed differential expression of transcripts associated with redox sensitive pathways such as NF-*k*B and MAPK signalling. Therefore gobies inhabiting the CO_2 seep could experience changes in cellular redox balance potentially affecting signal transduction (Zhang et al., 2016). Excessive ROS can also cause conformational changes and oxidative damage to all macromolecules including DNA, proteins, and lipids thereby affecting transmission of genetic information and resulting in intracellular accumulation of unfolded and misfolded proteins (Juan et al., 2021) which is cytotoxic (Hetz, 2012). Gobies however seem to be capable of minimising ocean acidification (OA) induced oxidative stress by differentially regulating transcripts involved in DNA damage response and DNA repair, unfolded protein response (UPR), and chaperones such as the heat shock protein (HSP) family. Upregulation of transcripts in fish from the CO_2 seep involved in cellular detoxification and redox regulation indicate that while they maybe under oxidative stress, they are capable of counteracting stress-induced damage. These are among the most universally conserved stress genes that are critical to the

cellular stress response machinery and hence high constitutive expression of these genes in fish from the CO_2 seep can confer increased cellular resistance to oxidative stress.

Immune response is another protective mechanism that is commonly differentially regulated in fish exposed to elevated CO_2 (de Souza et al., 2016; De Souza et al., 2014; Machado et al., 2020). We found an overall up-regulation of transcripts involved in the complement system, innate and adaptive immunity in gobies from the CO_2 seep site, particularly *nattectin* which showed a 142-fold increase in expression. Elevated levels of cellular stress can result in increased infections from opportunistic pathogens and hence activation of immune responses could be a protective mechanism to prevent potential infections in the face of increased physiological stress under conditions of OA (de Souza et al., 2016). Additionally, two hypoxia-inducible transcripts, *HIF1A* and *EGLN2* were upregulated in individuals from the CO_2 seep. While the primary function of these transcripts is to regulate cellular response to hypoxic conditions, their expression could also be enhanced in response to stress. Increased mRNA expression of *HIF1A* in response to hypercapnia has been previously reported under stable oxygen levels (Machado et al., 2020). These results indicate that gobies living in the naturally acidified waters of Vulcano Island are able to elicit physiological compensatory mechanisms to counter the cellular stress induced by prolonged exposure to elevated CO_2 levels.

Respiratory acidosis is also a common effect of elevated CO_2 exposure in fish (Ishimatsu et al., 2005) and hence maintaining pH homeostasis is crucial to ensure maintenance of proper cellular functioning in a high CO_2 environment (Heuer & Grosell, 2014). Carbonic anhydrase (CA), an enzyme which plays a key role in CO_2 excretion, acid-base balance, and ion-regulation was upregulated in gobies from the CO_2 seep, which could facilitate increased CO_2 excretion thereby preventing acidosis and also enhance transport of O_2 to the brain (Rummer & Brauner, 2011). Various other CA isoforms have been identified to be DE in

response to elevated CO_2 in the brain and gills of fish (Heuer & Grosell, 2014; Kang et al., 2022; Mittermayer et al., 2019; Monroe et al., 2021) suggesting that high CO_2 levels affect the expression and activity of CA. Additionally, transcripts encoding potassium channel transporters, and a Na^+/K^+ transporting ATPase were DE in the gobies. Acid-base balance and ion homeostasis in fish are closely linked processes as transport of H^+ and HCO_3^- ions are coupled to K^+, Na^+ and Cl^- transport (Gilmour & Perry, 2009). Potassium channels have been implicated to be involved in CO_2 chemoreception and sensing acid-base disturbance in zebrafish (Qin et al., 2010). The transcripts (*KCNAB3*, *KCNK10* and *KCNJ10*) encoding potassium channel proteins that were DE in our gobies could potentially be involved in enabling gobies living in the CO_2 seep to sense ambient CO_2 levels and in turn trigger downstream homeostatic responses to restore internal acid-base status. Additionally, the increased expression of *ATP1A1* in fish from the CO_2 seep may play an important role in pH homeostasis as the sodium and chloride gradient created by this enzyme affects the activity of other key secondary transporters involved in pH regulation such as NKCC, Na^+/H^+ exchangers and Na^+/ HCO_3^- cotransporters (Casey et al., 2010; Singh Jaggi et al., 2015; Tresguerres et al., 2020). Transcripts involved in actin cytoskeleton organisation was also DE which combined with DE of Ras GTPases, which are involved in remodelling the cytoskeleton, mentioned above could imply changes in membrane-cytoskeleton interactions in individuals from the CO_2 seep, in turn affecting membrane transport (de Curtis & Meldolesi, 2012). Therefore gobies inhabiting the naturally acidified waters of Vulcano Island can buffer pH changes by regulating ion transport to maintain cellular homeostasis in response to the altered sea-water chemistry in the CO_2 seep.

The pH buffering processes, however, can result in altered ionic composition which in turn causes neurological effects possibly due to reversal of γ-aminobutyric acid (GABA) receptor activity from inhibitory to excitatory (Heuer et al., 2016; Hübner & Holthoff, 2013;

Kang et al., 2022; Nilsson et al., 2012; Schunter et al., 2019). We found two transcripts *SLC6A13* and *GABAT* regulating the turnover rate of extracellular GABA to be DE. The increased expression of these transcripts could facilitate removal of excess GABA thereby minimising GABA receptor activation. Given that GABA is not prone to enzymatic degradation, upregulation of these transcripts could be an adaptive response to regulate GABAergic signalling. Furthermore, upregulation of the glutamate metabotropic receptor 4 (*GRM4*) in fish from the CO_2 seep could be a compensatory response to reduce presynaptic GABA release (Bobeck et al., 2014; Flor et al., 1995). Additionally, transcripts involved in the transport of chloride ions, whose concentration gradient influences GABA activity, were DE, which could potentially play an important role in restoring ionic gradients across neuronal membranes (Jentsch et al., 2002). Particularly, the downregulation of *SLC12A1* in the gobies could be an adaptive response to restore GABA function (Grosell, 2011; Hübner & Holthoff, 2013) similar to what has been observed with the potassium-chloride co-transporter 2 (KCC2) gene in the damselfish *Acanthochromis polyacanthus* exposed to elevated CO_2 (Schunter et al., 2018). Maintenance of a stable ionic environment of the brain ensuring proper neural functioning is also dependent on the activity of the blood-brain barrier (BBB) (Tresguerres & Hamilton, 2017). Transcripts encoding claudins, which are structural components of the tight junctions in the BBB and involved in regulating permeability of various ions (Kolosov et al., 2013), were upregulated in fish from CO_2 seep further suggesting regulation of ion transport in the brain. Therefore gobies inhabiting the naturally acidified waters of Vulcano Island are capable of buffering pH changes and maintaining physiological homeostasis while also potentially mitigating any negative consequences on GABA neurotransmission due to altered ionic gradients across neuronal membranes.

Nearly all physiological processes including ion and pH homeostasis are regulated by the circadian clock enabling organisms to anticipate and respond to environmental changes

which is evolutionarily advantageous (Ayyar & Sukumaran, 2021; Dmitriev & Mangel, 2000; Prokkola & Nikinmaa, 2018). All transcripts involved in the core circadian transcriptional machinery were differentially expressed between gobies from the CO_2 seep and control sites. Changes in the expression of circadian genes in response to elevated CO_2, have been suggested to result in an amplitude or phase shift of the circadian clock providing an adaptive advantage by increasing flexibility of physiological processes such as acid-base regulation and energy metabolism (Kang et al., 2022; Lee et al., 2021; Schunter et al., 2016, 2021). CO_2 induced changes in the circadian rhythm have been repeatedly observed across various species and are possibly linked to changes in GABA receptor activity (Lee et al., 2021). Given the ubiquity of the circadian clock, there is a need for further research to understand the biological implications of the effect of elevated CO_2 on the circadian rhythm and its impacts on downstream physiological processes. We found evidence of potential circadian regulation of metabolism in gobies in response to elevated environmental CO_2. *NOCT* an immediate early gene with deadenylase activity was upregulated in gobies from CO_2 seep. *NOCT* functions as a mediator of post-transcriptional circadian regulation of metabolic processes and can also respond to changes in the external environment (Stubblefield et al., 2012). Increased expression of *NOCT* in individuals from the CO_2 seep combined with DE of all core circadian genes suggest rhythmic regulation of energy metabolism potentially enabling gobies to meet the increased energetic demands of living in an acidified environment.

The constant need to maintain acid-base balance and homeostasis while minimising oxidative stress in a high CO_2 environment can be energetically expensive (Cattano et al., 2018; Kelly & Hofmann, 2013; Strader et al., 2020). We found an overall increase in expression of transcripts involved in ATP production and downregulation of transcripts involved in fatty-acid biosynthesis, an anabolic process. Acetyl-CoA is a key metabolite that intersects with glycolysis, TCA cycle, fatty acid beta-oxidation and fatty acid synthesis (Currais et al., 2019).

Therefore, downregulation of transcripts involved in fatty acid synthesis could facilitate allocation of acetyl-CoA for use in energy producing metabolic pathways. This in addition to up-regulation of *SLC16A1* and *SLC25A22*, which are also involved in transport of key metabolic intermediates, suggests that gobies selectively regulate metabolic pathways to ensure increased energy production. Additionally, all transcripts encoding hexose/glucose transporters, except for *SLC45A1* which is a sugar-H^+ symporter potentially facilitating H^+ import into neuronal cells (Shimokawa et al., 2002), were also upregulated in gobies from the CO_2 seep which could facilitate increased availability of glucose in the brain. This is particularly important as glucose is the primary substrate for energy production in the brain. Therefore, gobies have potentially acclimated to a high CO_2 environment by constitutively expressing genes involved in increased energy production. Changes in the energy budgets of fish living in acidified waters could result in physiological trade-offs (Kelly & Hofmann, 2013). However, the increased metabolic capacity of gobies combined with CO_2 driven enrichment of key trophic resources at the CO_2 seep (Nagelkerken et al., 2015; Tiralongo et al., 2020) potentially enables them to meet their energy requirements for maintaining pH homeostasis and mitigating any potential cellular damage due to elevated CO_2 levels without a significant trade-off in the energy available for other biological processes. In fact the CO_2 vents have a higher abundance of gobies compared to nearby control sites (Nagelkerken et al., 2015; Spatafora et al., in press). The CO_2 seeps in White Island which experience a similar habitat shift with net resource enrichment also have a higher abundance of the common triplefin *F. lapillum* and a similar increase in metabolic capacity was also reported for these fish (Nagelkerken et al., 2015; Petit-Marty et al., 2021). Therefore, gobies similar to the common triplefin might benefit from the habitat shifts at CO_2 seeps and show an adaptive response to prolonged CO_2 exposure with an overall increase in metabolism.

Our findings show molecular signatures of acclimation in the anemone goby to the acidified waters of Vulcano Island. The gobies effectively regulate disturbances in acid-base balance, maintain cellular homeostasis and mitigate OA induced oxidative stress. Gobies also showed potential adaptive mechanisms to restore GABA function, which is known to be disrupted as a consequence of altered ionic composition due to pH buffering processes. Although these processes can be energetically expensive potentially resulting in trade-offs, the overall increase in metabolism seen in gobies from the CO_2 seep suggests that they meet the increased energy demands associated with living in an acidified environment. This could be further facilitated by net trophic resource enrichment at the CO_2 seep. The increased population density of gobies at the CO_2 seep in Vulcano Island reported in previous studies combined with evidence of acclimation to OA found in our study indicates that gobies can effectively mitigate the behavioural effects of elevated CO_2. If gobies undergo adaptive selection like what has been seen in other species inhabiting CO_2 seeps, they might be able to endure future OA conditions.

Author contributions

AM conceived the project with input from CS and TR. AM conducted the fieldwork and dissected the fish. SV provided the funding for fieldwork. SS and CS carried out the RNA extractions, designed the RNA-Seq analysis pipeline and drafted the manuscript. All authors contributed to data interpretation and editing the manuscript. All authors read and approved the manuscript.

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Ethics

All sample collection were carried out in accordance with the institutional and national law guidelines concerning the use of animals in research.

Competing financial interests

All authors declare they have no competing interests.

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Data accessibility

RNA-seq raw sequences and the *de novo* assembled transcriptome assemblies have been deposited in NCBI under BioProject PRJNA869880.

References

Allan, B. J. M., Miller, G. M., Mccormick, M. I., Domenici, P., & Munday, P. L. (2014). Parental effects improve escape performance of juvenile reef fish in a high-CO_2 world. *Proceedings of the Royal Society B: Biological Sciences*, *281*(1777). https://doi.org/10.1098/RSPB.2013.2179

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Andrews, S. (2010). FastQC: a quality control tool for high throughput sequence data.

Apweiler, R. (2004). UniProt: the Universal Protein knowledgebase. *Nucleic Acids Research*, *32*(90001), 115D – 119. https://doi.org/10.1093/nar/gkh131

Ayyar, V. S., & Sukumaran, S. (2021). Circadian rhythms: influence on physiology, pharmacology, and therapeutic interventions. *Journal of Pharmacokinetics and Pharmacodynamics*, *48*(3), 321–338. https://doi.org/10.1007/S10928-021-09751-2

Bindoff, N. L., Cheung, W. W. L., Kairo, J. G., Arístegui, J., Guinder, V. A., Hallberg, R., Williamson, P. (2019). Chapter 5: Changing Ocean, Marine Ecosystems, and Dependent Communities - Special Report on the Ocean and Cryosphere in a Changing Climate. Retrieved June 29, 2022, from https://www.ipcc.ch/srocc/chapter/chapter-5/

Bobeck, E. N., Chen, Q., Morgan, M. M., & Ingram, S. L. (2014). Contribution of Adenylyl Cyclase Modulation of Pre- and Postsynaptic GABA Neurotransmission to Morphine Antinociception and Tolerance. *Neuropsychopharmacology 2014 39:9*, *39*(9), 2142–2152. https://doi.org/10.1038/npp.2014.62

Brauner, C. J., Shartau, R. B., Damsgaard, C., Esbaugh, A. J., Wilson, R. W., & Grosell, M. (2019). Acid-base physiology and CO_2 homeostasis: Regulation and compensation in response to elevated environmental CO_2. In *Fish Physiology* (Vol. 37). Academic Press. https://doi.org/10.1016/BS.FP.2019.08.003

Bresolin de Souza, K., Asker, N., Jönsson, E., Förlin, L., & Sturve, J. (2016). Increased activity of lysozyme and complement system in Atlantic halibut exposed to elevated CO_2 at six different temperatures. *Marine Environmental Research*, *122*, 143–147. https://doi.org/10.1016/J.MARENVRES.2016.09.005

Bushmanova, E., Antipov, D., Lapidus, A., Suvorov, V., & Prjibelski, A. D. (2016). RnaQUAST: A quality assessment tool for de novo transcriptome assemblies. *Bioinformatics*, *32*(14), 2210–2212. https://doi.org/10.1093/bioinformatics/btw218

Bushnell, B. (2014). BBMap: A Fast, Accurate, Splice-Aware Aligner. United States. Retrieved from https://www.osti.gov/servlets/purl/1241166

Cabau, C., Escudié, F., Djari, A., Guiguen, Y., Bobe, J., & Klopp, C. (2017). Compacting and correcting Trinity and Oases RNA-Seq de novo assemblies. *PeerJ*, *2017*(2). https://doi.org/10.7717/peerj.2988

Casey, J. R., Grinstein, S., & Orlowski, J. (2009). *Sensors and regulators of intracellular pH*. *Nature reviews Molecular cell biology*, *11*(1), 50-61. https://doi.org/10.1038/nrm2820

Cattano, C., Calò, A., Di Franco, A., Firmamento, R., Quattrocchi, F., Sdiri, K., Milazzo, M. (2017). Ocean acidification does not impair predator recognition but increases juvenile growth in a temperate wrasse off CO_2 seeps. *Marine Environmental Research*, *132*, 33-40. https://doi.org/10.1016/j.marenvres.2017.10.013

Cattano, C., Claudet, J., Domenici, P., & Milazzo, M. (2018). Living in a high CO_2 world: a global meta-analysis shows multiple trait-mediated fish responses to ocean acidification. *Ecological Monographs*, *88*(3), 320–335. https://doi.org/10.1002/ECM.1297

Cattano, C., Giomi, F., & Milazzo, M. (2016). Effects of ocean acidification on embryonic respiration and development of a temperate wrasse living along a natural CO_2 gradient. *Conservation Physiology*, *4*(1). https://doi.org/10.1093/CONPHYS/COV073

Chen, S., Zhou, Y., Chen, Y., & Gu, J. (2018). Fastp: An ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics*, *34*(17), i884–i890. https://doi.org/10.1093/bioinformatics/bty560

Connell, S. D., Kroeker, K. J., Fabricius, K. E., Kline, D. I., & Russell, B. D. (2013). The other ocean acidification problem: CO_2 as a resource among competitors for ecosystem dominance. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *368*(1627). https://doi.org/10.1098/RSTB.2012.0442

Currais, A., Huang, L., Goldberg, J., Petrascheck, M., Ates, G., Pinto-Duarte, A., Maher, P.

(2019). Elevating acetyl-CoA levels reduces aspects of brain aging. *ELife*, *8*. https://doi.org/10.7554/ELIFE.47866

Davidson, N. M., & Oshlack, A. (2014). Corset: Enabling differential gene expression analysis for de novo assembled transcriptomes. *Genome Biology*, *15*(7), 410. https://doi.org/10.1186/s13059-014-0410-6

de Curtis, I., & Meldolesi, J. (2012). Cell surface dynamics - how Rho GTPases orchestrate the interplay between the plasma membrane and the cortical cytoskeleton. *Journal of Cell Science*, *125*(Pt 19), 4435–4444. https://doi.org/10.1242/JCS.108266

De Souza, K. B., Jutfelt, F., Kling, P., Förlin, L., & Sturve, J. (2014). Effects of Increased CO_2 on Fish Gill and Plasma Proteome. *PLOS ONE*, *9*(7), e102901. https://doi.org/10.1371/JOURNAL.PONE.0102901

Dmitriev, A. V., & Mangel, S. C. (2000). A circadian clock regulates the pH of the fish retina. *The Journal of Physiology*, *522*(Pt 1), 77. https://doi.org/10.1111/J.1469-7793.2000.0077M.X

Domenici, P., Allan, B., McCormick, M. I., & Munday, P. L. (2012). Elevated carbon dioxide affects behavioural lateralization in a coral reef fish. *Biology Letters*, *8*(1), 78–81. https://doi.org/10.1098/RSBL.2011.0591

Doney, S. C., Fabry, V. J., Feely, R. A., & Kleypas, J. A. (2009). Ocean acidification: The other CO_2 problem. *Annual Review of Marine Science*, *1*, 169–192. https://doi.org/10.1146/annurev.marine.010908.163834

Doney, S. C., Ruckelshaus, M., Emmett Duffy, J., Barry, J. P., Chan, F., English, C. A., Talley, L. D. (2012). Climate change impacts on marine ecosystems. *Annual Review of Marine Science*, *4*, 11–37. https://doi.org/10.1146/annurev-marine-041911-111611

Esbaugh, A. J. (2017). Physiological implications of ocean acidification for marine fish: emerging patterns and new insights. *Journal of Comparative Physiology B 2017 188:1*, *188*(1), 1–13. https://doi.org/10.1007/S00360-017-1105-6

Fabricius, K. E., Langdon, C., Uthicke, S., Humphrey, C., Noonan, S., De'ath, G., Lough, J. M. (2011). Losers and winners in coral reefs acclimatized to elevated carbon dioxide concentrations. *Nature Climate Change 2011 1:3*, *1*(3), 165–169. https://doi.org/10.1038/NCLIMATE1122

Fabry, V. J., Seibel, B. A., Feely, R. A., & Orr Fabry, J. C. (2008). *Impacts of ocean acidification on marine fauna and ecosystem processes*. Retrieved from https://academic.oup.com/icesjms/article/65/3/414/789605

Ferrari, M. C. O., Dixson, D. L., Munday, P. L., Mccormick, M. I., Meekan, M. G., Sih, A., & Chivers, D. P. (2011). Intrageneric variation in antipredator responses of coral reef fishes affected by ocean acidification: implications for climate change projections on marine communities. *Global Change Biology*, *17*(9), 2980–2986. https://doi.org/10.1111/J.1365-2486.2011.02439.X

Ferrari, M. C. O., Mccormick, M. I., Munday, P. L., Meekan, M. G., Dixson, D. L., Lönnstedt, O., & Chivers, D. P. (2012). Effects of ocean acidification on visual risk assessment in coral reef fishes. *Functional Ecology*, *26*(3), 553–558. https://doi.org/10.1111/j.1365-2435.2011.01951.x

Flor, P. J., Lukic, S., Rüegg, D., Leonhardt, T., Knöpfel, T., & Kühn, R. (1995). Molecular cloning, functional expression and pharmacological characterization of the human metabotropic glutamate receptor type 4. *Neuropharmacology*, *34*(2), 149–155. https://doi.org/10.1016/0028-3908(94)00149-M

Galachyants, Y. P., Zakharova, Y. R., Volokitina, N. A., Morozov, A. A., Likhoshway, Y. V.,

& Grachev, M. A. (2019). De novo transcriptome assembly and analysis of the freshwater araphid diatom *Fragilaria radians*, Lake Baikal. *Scientific Data*, *6*(1), 1–11. https://doi.org/10.1038/s41597-019-0191-6

Gilmour, K. M., & Perry, S. F. (2009). Carbonic anhydrase and acid-base regulation in fish. *Journal of Experimental Biology*, *212*(11), 1647–1661. https://doi.org/10.1242/jeb.029181

Gö Tz, S., García-Gó Mez, J. M., Terol, J., Williams, T. D., Nagaraj, S. H., Nueda, M. J., Conesa, A. (2008). High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic Acids Research*, *36*(10), 3420–3435. https://doi.org/10.1093/nar/gkn176

Grabherr, M. G. ., Brian J. Haas, Moran Yassour Joshua Z. Levin, Dawn A. Thompson, Ido Amit, Xian Adiconis, Lin Fan, Raktima Raychowdhury, Qiandong Zeng, Zehua Chen, Evan Mauceli, Nir Hacohen, Andreas Gnirke, Nicholas Rhind, Federica di Palma, Bruce W., N., & Friedman, and A. R. (2013). Trinity: reconstructing a full-length transcriptome without a genome from RNA-Seq data. *Nature Biotechnology*, *29*(7), 644–652. https://doi.org/10.1038/nbt.1883.Trinity

Grosell, M. (2011). Intestinal anion exchange in marine teleosts is involved in osmoregulation and contributes to the oceanic inorganic carbon cycle. *Acta Physiologica (Oxford, England)*, *202*(3), 421–434. https://doi.org/10.1111/j.1748-1716.2010.02241.x

Haas, B. J., Papanicolaou, A., Yassour, M., Grabherr, M., Philip, D., Bowden, J., Regev, A. (2013). De novo transcript sequence recostruction from RNA-Seq: reference generation and analysis with Trinity. In *Nature protocols* (Vol. 8). https://doi.org/10.1038/nprot.2013.084.De

Hall-Spencer, J. M., Rodolfo-Metalpa, R., Martin, S., Ransome, E., Fine, M., Turner, S. M.,

Buia, M. C. (2008). Volcanic carbon dioxide vents show ecosystem effects of ocean acidification. *Nature 2008 454:7200*, *454*(7200), 96–99. https://doi.org/10.1038/NATURE07051

Hamilton, S. L., Logan, C. A., Fennie, H. W., Sogard, S. M., Barry, J. P., Makukhov, A. D., Bernardi, G. (2017). Species-Specific Responses of Juvenile Rockfish to Elevated pCO_2: From Behavior to Genomics. *PLOS ONE*, *12*(1), e0169670. https://doi.org/10.1371/JOURNAL.PONE.0169670

Hamilton, T. J., Holcombe, A., & Tresguerres, M. (2014). CO_2-induced ocean acidification increases anxiety in Rockfish via alteration of GABAA receptor functioning. *Proceedings of the Royal Society B: Biological Sciences*, *281*(1775). https://doi.org/10.1098/RSPB.2013.2509

Hetz, C. (2012). The unfolded protein response: controlling cell fate decisions under ER stress and beyond. *Nature Reviews. Molecular Cell Biology*, *13*(2), 89–102. https://doi.org/10.1038/NRM3270

Heuer, R M, Welch, M. J., Rummer, J. L., Munday, P. L., & Grosell, & M. (2016). Altered brain ion gradients following compensation for elevated CO_2 are linked to behavioural alterations in a coral reef fish. *Nature Publishing Group*. https://doi.org/10.1038/srep33216

Heuer, Rachael M., & Grosell, M. (2014). Physiological impacts of elevated carbon dioxide and ocean acidification on fish. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, *307*(9), R1061–R1084. https://doi.org/10.1152/AJPREGU.00064.2014

Heuer, Rachael M., Hamilton, T. J., & Nilsson, G. E. (2019). The physiology of behavioral impacts of high CO_2. *Fish Physiology*, *37*, 161–194.

https://doi.org/10.1016/BS.FP.2019.08.002

Hofmann, G. E., Barry, J. P., Edmunds, P. J., Gates, R. D., Hutchins, D. A., Klinger, T., & Sewell, M. A. (2010). The effect of Ocean acidification on calcifying organisms in marine ecosystems: An organism-to-ecosystem perspective. *Annual Review of Ecology, Evolution, and Systematics*, *41*, 127–147. https://doi.org/10.1146/annurev.ecolsys.110308.120227

Howe, K. L., Achuthan, P., Allen, J., Allen, J., Alvarez-Jarreta, J., Amode, M. R., Flicek, P. (2021). Ensembl 2021. *Nucleic Acids Research*, *49*(2). https://doi.org/10.1093/nar/gkaa942

Hübner, C. A., & Holthoff, K. (2013). Anion transport and GABA signaling. *Frontiers in Cellular Neuroscience*, *0*(OCT), 177. https://doi.org/10.3389/FNCEL.2013.00177/BIBTEX

Ishimatsu, A., Hayashi, M., Lee, K. S., Kikkawa, T., & Kita, J. (2005). Physiological effects on fishes in a high-CO_2 world. *Journal of Geophysical Research: Oceans*, *110*(C9), 1–8. https://doi.org/10.1029/2004JC002564

Jentsch, T. J., Stein, V., Weinreich, F., & Zdebik, A. A. (2002). Molecular structure and physiological function of chloride channels. *Physiological Reviews*, *82*(2), 503–568. https://doi.org/10.1152/physrev.00029.2001

Juan, C. A., de la Lastra, J. M. P., Plou, F. J., & Pérez-Lebeña, E. (2021). The Chemistry of Reactive Oxygen Species (ROS) Revisited: Outlining Their Role in Biological Macromolecules (DNA, Lipids and Proteins) and Induced Pathologies. *International Journal of Molecular Sciences*, *22*(9). https://doi.org/10.3390/IJMS22094642

Kang, J., Nagelkerken, I., Rummer, J. L., Rodolfo-Metalpa, R., Munday, P. L., Ravasi, T., &

Schunter, C. (2022). Rapid evolution fuels transcriptional plasticity to ocean acidification. *Global Change Biology*. https://doi.org/10.1111/GCB.16119

Kelly, M. W., & Hofmann, G. E. (2013). Adaptation and the physiology of ocean acidification. *Functional Ecology*, *27*(4), 980–990. https://doi.org/10.1111/J.1365-2435.2012.02061.X

Kolosov, D., Bui, P., Chasiotis, H., & Kelly, S. P. (2013). Claudins in teleost fishes. *Tissue Barriers*, *1*(3), e25391. https://doi.org/10.4161/TISB.25391

Kroeker, K. J., Kordas, R. L., Crim, R. N., & Singh, G. G. (2010). Meta-analysis reveals negative yet variable effects of ocean acidification on marine organisms. *Ecology Letters*, *13*(11), 1419–1434. https://doi.org/10.1111/j.1461-0248.2010.01518.x

Lai, F., Fagernes, C. E., Jutfelt, F., Nilsson, G. E., & Cooke, S. (2016). Expression of genes involved in brain GABAergic neurotransmission in three-spined stickleback exposed to near-future CO_2. *Conservation physiology*, *4*(1). https://doi.org/10.1093/conphys/cow068

Langmead, B., & Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature Methods*, *9*(4), 357–359. https://doi.org/10.1038/nmeth.1923

Lee, D.-W., Song, J. A., Park, H.-S., Cheol, ·, & Choi, Y. (2021). Circadian Rhythm Disturbances Due to Exposure to Acidified Conditions and Different Photoperiods in Juvenile Olive Flounder (*Paralichthys olivaceus*). *Ocean Science Journal*, *56*, 198–206. https://doi.org/10.1007/s12601-021-00018-y

Li, B., & Dewey, C. N. (2014). RSEM: Accurate transcript quantification from RNA-seq data with or without a reference genome. In *Bioinformatics: The Impact of Accurate Quantification on Proteomic and Genetic Analysis and Research* (pp. 41–74). Apple

Academic Press. https://doi.org/10.1201/b16589

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, *15*(12), 1–21. https://doi.org/10.1186/s13059-014-0550-8

Machado, M., Arenas, F., Svendsen, J. C., Azeredo, R., Pfeifer, L. J., Wilson, J. M., & Costas, B. (2020). Effects of Water Acidification on Senegalese Sole *Solea senegalensis* Health Status and Metabolic Rate: Implications for Immune Responses and Energy Use. *Frontiers in Physiology*, *11*, 26. https://doi.org/10.3389/FPHYS.2020.00026/BIBTEX

Manni, M., Berkeley, M. R., Seppey, M., Simão, F. A., & Zdobnov, E. M. (2021). BUSCO Update: Novel and Streamlined Workflows along with Broader and Deeper Phylogenetic Coverage for Scoring of Eukaryotic, Prokaryotic, and Viral Genomes. *Molecular Biology and Evolution*, *38*(10), 4647–4654. https://doi.org/10.1093/MOLBEV/MSAB199

Milazzo, M., Cattano, C., Alonzo, S. H., Foggo, A., Gristina, M., Rodolfo-Metalpa, R., Hall-Spencer, J. M. (2016). Ocean acidification affects fish spawning but not paternity at CO_2 seeps. *Proceedings of the Royal Society B: Biological Sciences*, *283*(1835). https://doi.org/10.1098/rspb.2016.1021

Mirasole, A., Gillanders, B. M., Reis-Santos, P., Grassa, F., Capasso, G., Scopelliti, G., Vizzini, S. (2017). The influence of high pCO_2 on otolith shape, chemical and carbon isotope composition of six coastal fish species in a Mediterranean shallow CO_2 vent. *Marine Biology*, *164*(9). https://doi.org/10.1007/s00227-017-3221-y

Mirasole, A., Scopelliti, G., Tramati, C., Signa, G., Mazzola, A., & Vizzini, S. (2021). Evidences on alterations in skeleton composition and mineralization in a site-attached fish under naturally acidified conditions in a shallow CO_2 vent. *Science of The Total*

Environment, *761*, 143309. https://doi.org/10.1016/J.SCITOTENV.2020.143309

Mirasole, A., Signa, G., Gianguzza, P., Bonaviri, C., Mazzola, A., & Vizzini, S. (2019). Fish assemblages cope with ocean acidification in a shallow volcanic CO_2 vent benefiting from an adjacent recovery area. *Marine Environmental Research*. https://doi.org/10.1016/j.marenvres.2019.104851

Mittermayer, H., Stiasny, M. H., Puvanendran, V., Chierici, M., Jentoft, S., & Reusch, B. H. (2019). Transcriptome profiling reveals exposure to predicted end-of-century ocean acidification as a stealth stressor for Atlantic cod larvae. *Scientific Reports*, *9(1)*, 1–11. https://doi.org/10.1038/s41598-019-52628-1

Monroe, A. A., Schunter, C., Welch, M. J., Munday, P. L., & Ravasi, T. (2021). Molecular basis of parental contributions to the behavioural tolerance of elevated pCO_2 in a coral reef fish. *Proceedings of the Royal Society B: Biological Sciences*, *288*(1964). https://doi.org/10.1098/rspb.2021.1931

Munday, P. L. (2014). Transgenerational acclimation of fishes to climate change and ocean acidification. *F1000Prime Reports*, *6*. https://doi.org/10.12703/P6-99

Munday, P. L., Cheal, A. J., Dixson, D. L., Rummer, J. L., & Fabricius, K. E. (2014). Behavioural impairment in reef fishes caused by ocean acidification at CO_2 seeps. *Nature Climate Change*, *4*(6), 487–492. https://doi.org/10.1038/nclimate2195

Munday, P. L., Dixson, D. L., McCormick, M. I., Meekan, M., Ferrari, M. C. O., & Chivers, D. P. (2010). Replenishment of fish populations is threatened by ocean acidification. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(29), 12930–12934. https://doi.org/10.1073/PNAS.1004519107/SUPPL_FILE/PNAS.201004519SI.PDF

Munday, P. L., McCormick, M. I., Meekan, M., Dixson, D. L., Watson, S.-A., Chivers, D. P., & Ferrari, M. C. O. (2013). Selective mortality associated with variation in CO_2 tolerance in a marine fish. *Ocean Acidification*, *1*, 1–5. https://doi.org/10.2478/OAC-2012-0001

Munday, P. L., Pratchett, M. S., Dixson, D. L., Donelson, J. M., Endo, G. G. K., Reynolds, A. D., & Knuckey, R. (2013). Elevated CO_2 affects the behavior of an ecologically and economically important coral reef fish. *Marine Biology*, *160*(8), 2137–2144. https://doi.org/10.1007/S00227-012-2111-6/FIGURES/2

Nagelkerken, I., Russell, B. D., Gillanders, B. M., Connell, S. D., & Kong SAR, H. (2015). Ocean acidification alters fish populations indirectly through habitat modification. *Nature Climate Change*, *6(*1), 89-93. https://doi.org/10.1038/NCLIMATE2757

Nilsson, G. E., Dixson, D. L., Domenici, P., McCormick, M. I., Sørensen, C., Watson, S. A., & Munday, P. L. (2012). Near-future carbon dioxide levels alter fish behaviour by interfering with neurotransmitter function. *Nature Climate Change*, *2*(3), 201–204. https://doi.org/10.1038/nclimate1352

OmicsBox – Bioinformatics Made Easy, BioBam Bioinformatics, March 3. (2019). https://www.biobam.com/omicsbox

Petit-Marty, N., Nagelkerken, I., Connell, S. D., & Schunter, C. (2021). Natural CO_2 seeps reveal adaptive potential to ocean acidification in fish. *Evolutionary Applications*, *14*(7), 1794–1806. https://doi.org/10.1111/eva.13239

Pizzino, G., Irrera, N., Cucinotta, M., Pallio, G., Mannino, F., Arcoraci, V., Bitto, A. (2017). Oxidative Stress: Harms and Benefits for Human Health. *Oxidative Medicine and Cellular Longevity*, *2017*. https://doi.org/10.1155/2017/8416763

Poli, G., Leonarduzzi, G., Biasi, F., & Chiarpotto, E. (2012). Oxidative Stress and Cell Signalling. *Current Medicinal Chemistry*, *11*(9), 1163–1182. https://doi.org/10.2174/0929867043365323

Prokkola, J. M., & Nikinmaa, M. (2018). Circadian rhythms and environmental disturbances - Underexplored interactions. *Journal of Experimental Biology*, *221*(16). https://doi.org/10.1242/JEB.179267/19339

Qin, Z., Lewis, J. E., & Perry, S. F. (2010). Zebrafish (*Danio rerio*) gill neuroepithelial cells are sensitive chemoreceptors for environmental CO_2. *The Journal of Physiology*, *588*(5), 861–872. https://doi.org/10.1113/JPHYSIOL.2009.184739

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research*, *41*(D1), 590–596. https://doi.org/10.1093/nar/gks1219

R Core Team. (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Retrieved from https://www.r-project.org/.

Radford, C. A., Collins, S. P., Munday, P. L., & Parsons, D. (2021). Ocean acidification effects on fish hearing. *Proceedings of the Royal Society B: Biological Sciences*, *288*(1946). https://doi.org/10.1098/rspb.2020.2754

Rummer, J. L., & Brauner, C. J. (2011). Plasma-accessible carbonic anhydrase at the tissue of a teleost fish may greatly enhance oxygen delivery: in vitro evidence in rainbow trout, *Oncorhynchus mykiss*. *Journal of Experimental Biology*, *214*(14), 2319–2328. https://doi.org/10.1242/JEB.054049

Sahraeian, S. M. E., Mohiyuddin, M., Sebra, R., Tilgner, H., Afshar, P. T., Au, K. F., … Lam, H. Y. K. (2017). Gaining comprehensive biological insight into the transcriptome by performing a broad-spectrum RNA-seq analysis. *Nature Communications*, *8*(1), 1–14. https://doi.org/10.1038/s41467-017-00050-4

Schunter, C., Jarrold, M. D., Munday, P. L., & Ravasi, T. (2021). Diel pCO_2 fluctuations alter the molecular response of coral reef fishes to ocean acidification conditions. *Molecular Ecology*, *30*(20), 5105–5118. https://doi.org/10.1111/mec.16124

Schunter, C., Ravasi, T., Munday, P. L., & Nilsson, G. E. (2019). Neural effects of elevated CO_2 in fish may be amplified by a vicious cycle. *Conservation Physiology*, *7*(1). https://doi.org/10.1093/CONPHYS/COZ100

Schunter, C., Welch, M. J., Nilsson, G. E., Rummer, J. L., Munday, P. L., & Ravasi, T. (2018). An interplay between plasticity and parental phenotype determines impacts of ocean acidification on a reef fish. *Nature Ecology & Evolution*, *2*(2), 334–342. https://doi.org/10.1038/s41559-017-0428-8

Schunter, C., Welch, M. J., Ryu, T., Zhang, H., Berumen, M. L., Nilsson, G. E., Ravasi, T. (2016). Molecular signatures of transgenerational response to ocean acidification in a species of reef fish. *Nature Climate Change*, *6*(11), 1014–1018. https://doi.org/10.1038/nclimate3087

Shimokawa, N., Okada, J., Haglund, K., Dikic, I., Koibuchi, N., & Miura, M. (2002). Past-A, a novel proton-associated sugar transporter, regulates glucose homeostasis in the brain. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *22*(21), 9160–9165. https://doi.org/10.1523/JNEUROSCI.22-21-09160.2002

Simpson, S. D., Munday, P. L., Wittenrich, M. L., Manassa, R., Dixson, D. L., Gagliano, M., & Yan, H. Y. (2011). Ocean acidification erodes crucial auditory behaviour in a marine

fish. *Biology Letters*, *7*(6), 917–920. https://doi.org/10.1098/RSBL.2011.0293

Singh Jaggi, A., Kaur, A., Bali, A., & Singh, N. (2015). Expanding Spectrum of Sodium Potassium Chloride Co-transporters in the Pathophysiology of Diseases. *Current Neuropharmacology*, *13*(3), 369-388.

Stiasny, M. H., Mittermayer, F. H., Göttler, G., Bridges, C. R., Falk-Petersen, I. B., Puvanendran, V., Clemmesen, C. (2018). Effects of parental acclimation and energy limitation in response to high CO_2 exposure in Atlantic cod. *Scientific Reports 2018 8:1*, *8*(1), 1–8. https://doi.org/10.1038/s41598-018-26711-y

Strader, M. E., Wong, J. M., & Hofmann, G. E. (2020). Ocean acidification promotes broad transcriptomic responses in marine metazoans: A literature survey. *Frontiers in Zoology*, *17*(1), 1–23. https://doi.org/10.1186/s12983-020-0350-9

Stubblefield, J. J., Terrien, J., & Green, C. B. (2012). Nocturnin: At the crossroads of clocks and metabolism. *Trends in Endocrinology and Metabolism*, *23*(7), 326–333. https://doi.org/10.1016/j.tem.2012.03.007

Sunday, J. M., Calosi, P., Dupont, S., Munday, P. L., Stillman, J. H., & Reusch, T. B. H. (2014). Evolution in an acidifying ocean. *Trends in Ecology & Evolution*, *29*(2), 117–125. https://doi.org/10.1016/J.TREE.2013.11.001

Thacker, C. E., & Roje, D. M. (2011). Phylogeny of Gobiidae and identification of gobiid lineages. *Systematics and Biodiversity*, *9*(4), 329–347. https://doi.org/10.1080/14772000.2011.629011

Tresguerres, M., Clifford, A. M., Harter, T. S., Roa, J. N., Thies, A. B., Yee, D. P., & Brauner, C. J. (2020). Evolutionary links between intra-and extracellular acid-base regulation in fish and other aquatic animals. *J. Exp. Zool*, *333*, 449–465.

https://doi.org/10.1002/jez.2367

Tresguerres, M., & Hamilton, T. J. (2017). Acid–base physiology, neurobiology and behaviour in relation to CO_2-induced ocean acidification. *Journal of Experimental Biology*, *220*(12), 2136–2148. https://doi.org/10.1242/JEB.144113

Tsang, H. H., Welch, M. J., Munday, P. L., Ravasi, T., & Schunter, C. (2020). Proteomic Responses to Ocean Acidification in the Brain of Juvenile Coral Reef Fish. *Frontiers in Marine Science*, *7*, 605. https://doi.org/10.3389/FMARS.2020.00605/BIBTEX

Vizzini, S., Martínez-Crego, B., Andolina, C., Massa-Gallucci, A., Connell, S. D., & Gambi, M. C. (2017). Ocean acidification as a driver of community simplification via the collapse of higher-order and rise of lower-order consumers. *Scientific Reports 2017 7:1*, *7*(1), 1–10. https://doi.org/10.1038/s41598-017-03802-w

Welch, M. J., & Munday, P. L. (2017). Heritability of behavioural tolerance to high CO_2 in a coral reef fish is masked by nonadaptive phenotypic plasticity. *Evolutionary Applications*, *10*(7), 682–693. https://doi.org/10.1111/eva.12483

Wood, D. E., & Salzberg, S. L. (2014). Kraken: Ultrafast metagenomic sequence classification using exact alignments. *Genome Biology*, *15*(3). https://doi.org/10.1186/gb-2014-15-3-r46

Zhang, J., Wang, X., Vikash, V., Ye, Q., Wu, D., Liu, Y., & Dong, W. (2016). ROS and ROS-Mediated Cellular Signaling. *Oxidative Medicine and Cellular Longevity*, *2016*. https://doi.org/10.1155/2016/4350965

Zunino, S., Canu, D. M., Zupo, V., & Solidoro, C. (2019). Direct and indirect impacts of marine acidification on the ecosystem services provided by coralligenous reefs and seagrass systems. *Global Ecology and Conservation*, *18*, e00625.

914 https://doi.org/10.1016/J.GECCO.2019.E00625