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Gills *de novo* assembly reveals oxidative stress, unfolded protein, and immune response on red cusk-eel (*Genypterus chilensis*) under thermal stress

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ABSTRACT

The heat waves on the South Pacific coast could lead to thermal stress in native fish. The red cusk-eel (*Genypterus chilensis*) is relevant for Chilean artisanal fisheries and aquaculture diversification. This study examined the effect of high-temperature stress in the gills of *G. chilensis* in control (14 °C) and high-temperature stress (19 °C) conditions. High-temperature stress induces a significant increase in gills cortisol levels. Additionally, oxidative damage was observed in gills (protein carbonylation and lipoperoxidation). RNA-seq data was used to build the first transcriptome assembly of gills in this species (23,656 annotated transcripts). A total of 1138 down-regulated and 1531 up-regulated transcripts were observed in response to high-temperature stress in gills. The enrichment analysis showed immune response and replication enriched processes (on down-regulated transcripts), and processes related to the folding of proteins, endoplasmic reticulum, and transporter activity (on up-regulated transcripts). The present study showed how gills could be affected by high-temperature stress.

1. Background

Poikilotherm fish are a broad group of vertebrates in which body temperature changes according to environmental temperatures (Sokolova, 2019), generating physiological changes in the fish's metabolism according to their environment (Xiong et al., 2022; Cominassi et al., 2022). In this sense, each fish species has its optimal water temperature range, including lower and upper critical limits, which could be related to the adaptation capacity to temperature change (Calosi et al., 2008). This adaptation capacity is relevant in teleost fish, considering the actual scenario of climate changes, which is associated with an increase in ocean means temperatures and increased intensity and frequency of extreme heat events (Montie et al., 2020; Dobretsov et al., 2019; Rahmstorf and Coumou, 2011; Meehl and Tebaldi, 2004). In the south hemisphere, these effects of extreme heat events are associated with higher intensity of El Niño–Southern Oscillation (ENSO), leading to water temperature increase, and therefore, the possible effect of this increase in marine fish species of the south Pacific coast (Wang et al., 2019).

Considering these effects on sea temperature in the short and long term is relevant to study the effects of these changes on relevant fish for the food chain. This is critical considering the necessity of animal food sources required to provide animal protein, representing marine organisms as a relevant protein source to supply this demand (Cochrane et al., 2012). As part of this effort to increase protein production from marine origins, we found aquaculture as a key source of marine protein. However, is predicted that increased temperature and heatwaves under climate change scenario increase stress, diseases, and mortalities and decrease productivity in aquaculture species (Barange et al., 2018). Aquaculture diversification represents a good alternative to contribute to providing new marine protein sources, requiring generating knowledge and technology to produce food using new native species. One of the species that has contributed to aquaculture diversification in Chile is the red cusk-eel (*Genypterus chilensis*), an endemic species of the South

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Pacific coast (Vega et al., 2012), living on rocky bottoms, and that is important for the artisanal fisheries (Vega et al., 2015), with more than 2000 tons of capture in 2022 for *Genypterus* species in Chile (SERNAPESCA, 2023). Additionally, this species presents a wide distribution from the south of Peru to the south of Chile, but with genetic studies showing a low level of migration between populations of the species (Gonzalez et al., 2019). However, despite the relevance of this species, the biological knowledge as well as the genomic resources are still limited.

In marine environments, as well as in open aquaculture systems, environmental conditions are relevant for the welfare of the fish (Ashley, 2007). One of the main environmental factors that could impact the fish biology is the sea temperature (Bartolini et al., 2015; Strowbridge et al., 2021). Change in sea temperature, including low or high temperature could lead to a stress response in the fish, generating a secondary and a tertiary response, with negative effect for the fish (Faught et al., 2016). One type of alteration at cellular level that could be induced by environmental stressors is the oxidative stress, in which an unbalance between oxidant agents and antioxidant could lead to oxidative damage in the cellular structures, with posterior negative effects for the cell and the organism (Baker et al., 2020; Betteridge, 2000). The effect of the stressor in the oxidative status is dependent of the type of stressor, species, tissue and developmental stage, among others (Adachi et al., 2009), as previously observed in the case of *G. chilensis* in liver, muscle and eggs (Dettleff et al., 2020b, 2021, 2022). In this sense, the gills represent a relevant tissue on the fish physiology, in which the gas exchange and nitrogenous waste compounds are interchange on marine environment, among others (Wong et al., 2023; Bollinger et al., 2016). Additionally, it has been observed that gills could play a role in the immune response of the fish (Thompsom, 2017), acting as a secondary lymphoid tissue through the lymphoid cell associated to gills, or gill-associated lymphoid tissue (GIALT) (Resseguier et al., 2020). Therefore, the direct interaction of gills with the environment made this tissue susceptible to change in marine water conditions, observing a modulation of oxidative status associated to environmental stressors in teleost fish (Sun et al., 2020). However, in species of *Genypterus* genus, no information exists related to the effect of environmental stressors in gills, neither the changes in oxidative status, immune modulation or stress response. The objective of this study was to determine the effect of high temperature in short periods simulating a scenario of sea heath waves, to assess the effect at stress response, oxidate status and immune modulation in *G. chilensis* in a relevant tissue as gills in this species.

2. Methods

2.1. High temperature assay and red cusk-eel sampling

To determine the effect of high temperature in gills, we use juveniles (immature) red cusk-eel (*G. chilensis*) of one year of age, with average weight of 680 g (±64 g) and average length of 59.5 cm (±3.3 cm), collected from the Center of Marine Research of Quintay (CIMARQ), located in Quintay, Valparaiso Region, Chile. Fish were maintained under natural photoperiod condition (12 h light:12 h dark) and fed daily with commercial pellet food. Fish were submitted to an acclimatization period to tank condition ten days before the experiment. Before the experimental assay, fish were separated on control (C; n = 6) and high temperature stress (S; n = 6) groups, with two replicated thanks per each experimental group at a density of 10 kg/m3. The experimental assay was performed by five days, with the C group maintained at an average water temperature of 14 °C (±0.8 °C) and the S group was maintained at 19 °C (±0.7 °C). Additionally, O_2 concentration, pH and salinity were monitored in tanks (7.3 ± 0.08 mg/L O_2; pH 7.9 ± 0.05; 34 psμ). This temperature in the S group was reached following a previous standardized thermal increase protocol in 24 h (Dettleff et al., 2020b). The high temperature was selected considering the heat waves on the northern Chilean coast according to SHOA data (SHOA, 2019), temperature that in previous studies we have observed that could generate thermal stress in *Genypterus* species (Dettleff et al., 2020b, 2021, 2022). After five days, fish were anesthetized for gill filament sampling for cortisol assay and then euthanized by anesthetic overdose with tricaine (300 mg/L). The gill tissue of the fish was sampled, cutting gill filament (20 mg of tissue) and stored at −80 °C for posterior protein quantification and cortisol analysis. Additional gill tissue was sampled and frozen in liquid nitrogen for oxidative stress assays, and also gill tissue was stored on RNAsave solution (Biological Industries, Cromwell, CT, USA) and maintained at −80 °C for posterior RNA sequencing.

2.2. Gills cortisol determination

To determine the effect of high temperature in physiological stress, cortisol levels were determined in gill filaments with the commercial Enzyme Immunoassay Kit (Cayman, MI, USA) following the manufacturer's recommendations. Gill tissue was homogenized on 120 μL of phosphate-buffered saline (PBS, pH = 7.3) and centrifugated, using supernatant for ELISA assay, according to the protocol described of (Gesto et al., 2015). Gill cortisol level was normalized by tissue protein content and expressed as ng/mg of protein. For this, protein content of gill tissue was extracted from homogenized tissue in 1 mL of lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40, and a protease inhibitor cocktail (Calbiochem, Billerica, MA, USA). Protein quantification was performed with Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA), which has been previously used successfully to normalize corticosteroids by protein concentration (Schloms and Swart, 2014). All the spectrophotometry measurements for cortisol and protein quantification analysis were performed with the Epoch Spectrophotometer System (BioTek, VT, USA).

2.3. Oxidative stress determination in gills

To assess the effect of high temperature in gills at oxidative stress status, oxidative damage was determined at DNA, protein carbonylation and lipoperoxidation using commercially available kits. To evaluate oxidative stress in DNA, apurinic/apyrimidinic sites (AP sites) were determined in the genomic DNA of gills, extracted from 25 mg of tissue with DNAzol reagent (Invitrogen, CA, USA) following the manufacturer's protocol, and concentration and purity were determined by Epoch Spectrophotometer System (BioTek, VT, USA). The AP sites were then determined with the OxiSelect Oxidative DNA Damage Quantification Kit (Cell Biolabs, CA, USA) following the manufacturer's protocol. To determine protein carbonylation, total protein was extracted as previously indicated and quantified using the Pierce BCA Protein Assay Kit (Thermo Scientific, IL, USA). The carbonylation of proteins was then determined using the OxiSelect Protein Carbonyl Spectrophotometric Assay (Cell Biolabs, CA, USA) following the manufacturer's protocol. The lipoperoxidation was determined evaluating the quantification of hydroxynonenal (HNE) protein adducts in gills proteins, using the OxiSelect HNE Adduct Competitive ELISA Kit (Cell Biolabs, CA, USA), according to the manufacturer's instructions.

2.4. Gills RNA isolation, library preparation, sequencing and reads filtering

The stored gill tissue was used to RNA extraction using the TRIzol® reagent (Invitrogen, CA, USA) protocol, following manufacturer instructions. The RNA was quantified with Qubit® RNA quantification assay fluorometry (Invitrogen, Carlsbad, CA, USA) and integrity was determined, and samples selected (RQN ≥8) by Fragment Analyzer with Standard Sensitivity RNA analysis kit (Advanced Analytical Technologies, Fiorenzuola, Italy). The sequencing library was generated from 1 μg of total RNA using the Illumina RNA Sample Prep kit v2 (Illumina ®, San Diego, CA, USA), quantified by the Kapa Library Quantification kit

(Roche, Little Falls, NJ, USA) on a Stratagene MX3000P qPCR system (Stratagene, La Jolla, CA, USA) and library size was determined by Fragment Analyzer equipment, using the NGS fragment analysis kit (Advanced Analytical Technologies, Fiorenzuola, Italy). The sequencing was performed on a HiSeq 2500 (Illumina ®) platform with a pair-end sequencing (2 x 100 bp) on Macrogen Inc (Seoul, South Korea). The quality check of sequenced reads was determined with FastQC (Andrews, 2010), and then filtered with Trimmomatic v 0.22 (Bolger et al., 2014) to remove low-quality reads and bases, as well as remanent adapters sequences.

2.5. Transcriptome de novo assembly and annotation

The reads obtained from Trimmomatic from C and S groups were used as input for Trinity software v 2.1.1.6 (Grabherr et al., 2011) to build the gill *de novo* assembly of *G. chilensis*. The assembly parameters include a minimum contig size of 500 bp. The % GC and N50 statistic were determined for *de novo* assembly and orthologs were inferred on the transcriptome using the Benchmarking Universal Single-Copy Orthologs (BUSCO) software v5.4.4 (Simao et al., 2015) using *Vertebrata* and *Actinopterygi* databases as references. To identify Open Reading Frames (ORFs) in the assembly we run TransDecoder software v 5.5.0 (https://github.com/TransDecoder/TransDecoder/releases), and to reduce contig redundance we run CD-Hit-EST software v4.8.1 (Fu et al., 2012) in the transcripts of the *de novo* assembly.

The final set of contig of the assembly was annotated using NCBI Nr database (downloaded October 2022) with the BLASTx algorithm on local BLAST server with matrix BLOSUM62, and threshold of E-value 10e-5. The BLAST annotations were loaded on Blast2GO software (Conesa et al., 2005) for functional annotation of transcripts with Gene Ontology (GO) terms. After GO annotation, transcripts were annotated with KEGG Automatic Annotation Server (KAAS) (Moriya et al., 2007) using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway databases to identify KEGG Orthology (KO) annotations.

2.6. Differential expression analysis and enrichment analysis

The C and S filtered libraries were mapped against the annotated gill transcriptome of *G. chilensis* with CLC Genomics Workbench v.7.0.3 software using the following parameters: mismatch cost = 2, insertion cost = 3, deletion cost = 3, length fraction = 0.8, similarity fraction = 0.8. After mapping, the expression values from unique reads were retrieved and used as input for differential expression analysis with DESeq2 v 1.2.10 (Love et al., 2014) R package, to determine significant difference between C and S groups in gills of *G. chilensis* due to high temperature, considering differentially expressed transcripts those with an adjusted p-value (using the Benjamini and Hochberg method by default in DESeq2) of <0.01 and an absolute log2 fold change of >1. The list of differentially expressed transcripts between C and S groups was used to perform an enrichment analysis using the enrichment analysis tool implemented in the Blast2GO software (Conesa et al., 2005). Clustering and heatmap graph were generated using expression values in R and GO pathway enrichment plot was performed with SRPlot platform (https://www.bioinformatics.com.cn/en). Additionally, differentially expressed transcripts were used to identify represented KEGG pathways with the KO annotations using the KASS server (Moriya et al., 2007).

2.7. qPCR validation of differential expression

To validate RNA-seq differentially expressed data we selected fourteen transcripts. These transcripts include: *psmd7, psma3, gzma, mpv17l, muc5ac, aqp11, aqp3, pxmp2, mapk4, hsp70, hsp90, gst, sod1* and *hsp60*. The gene expression of these genes was obtained normalizing the expression of the target gene by the geometric mean of two reference genes previously validated in *G. chilensis* (Dettleff et al., 2020b, 2022) corresponding to *actb* and *taf12*, following the methodology described by (Vandesompele et al., 2002). The primer for each of these transcripts were designed with the online tool PrimerQuest™ Tool (Integrated DNA Technologies (IDT), Inc., Coralville, IA, USA) available at https://www.idtdna.com/SciTools, using the *de novo* assembled sequences of gill generated for *G. chilensis*. The description of primers, including sequence, size, Tm and efficiency are in Supplementary Table 1.

A total of 1 μg of gill RNA per sample was pre-trated with DNAse and then transcribed to cDNA with the QuantiTect® Reverse Transcription kit (Qiagen, TX, USA), following the manufacturer's protocol. The RT-qPCR were performed on a Stratagene MX3000P qPCR platform (Stratagene, La Jolla, CA, USA). Each qPCR reaction contained 100 ng of total cDNA, 200 nM of each primer, 7.5 μl of 2x Brilliant® II SYBR® Green master mix (Agilent Technologies, CA, USA) in a total volume of 15 μl. The qPCR program was: 2 min at 95 °C; 40 cycles of 30 s of denaturation at 95 °C; 30 s of annealing at 62 °C; 30 s of elongation at 72 °C. Additionally, at the end of the program, a melt curve was run to confirm the presence of a single qPCR product. Each sample was run in triplicate, including no-template control and no-RT control. To determine the efficiency of each primer, a standard curve with the $E = 10^{(-1/slope)}$-1 formula was build using serial dilutions (2-fold). The qPCR results were analyzed with the MxPro qPCR software (Agilent Technologies, CA, USA) and in compliance with the MIQE guidelines (Bustin et al., 2009).

2.8. Statistical analysis

To determine significant differences between C and S groups on gill cortisol determination, oxidative damage assays on DNA, proteins and lipoperoxidation (AP sites, carbonylation of proteins and HNE, respectively), and differentially expression of qPCR transcripts, a *t*-test was performed, with a significant threshold of $P < 0.05$. Pearson correlation coefficient between log2FC values of RNA-seq and qPCR of validated transcripts was determined. The statistical analysis was performed with GraphPad Prism v5.0 (GraphPad, San Diego, CA, USA).

3. Results

3.1. Cortisol and oxidative stress in gills in response to high temperature

To evaluate the effect of the increase of temperature by five days in stress status of *G. chilensis* in gills, we determined the level of gill cortisol normalized by protein content, as a useable measure to be applicable in

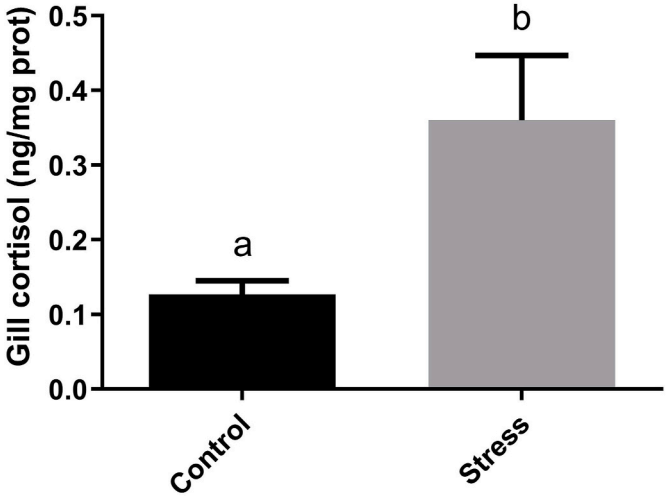


Fig. 1. Gill levels of cortisol in *Genypterus chilensis* under high temperature stress. Levels of cortisol in ng/mg protein in gills of control (C) and high temperature stress (S) groups. Bars represent means ± SEM. Different letters indicate significant differences between groups (p-value <0.05).

fish (Gesto et al., 2015). The high temperature generates a significant increase in the cortisol level in gill tissue of the S group (Fig. 1) (2.83-times respect to C group, $P < 0.05$).

In the case of oxidative stress, to evaluate the effect of high temperature in gills, we determined oxidative damage at DNA, measure as AP sites, damage in proteins as protein carbonylation, and lipoperoxidation, measure as HNE protein adducts. At DNA level the high temperature does not generate significant DNA damage in S group (Fig. 2A). However, at protein carbonylation, a significant increase was observed in the S group, indicating oxidative damage in proteins of gills (Fig. 2B) (2.21-times respect to C group, $P < 0.05$). Additionally, oxidative damage also was observed at lipoperoxidation, with a significant increase in HNE protein adducts of gills in S group in response to high temperature (Fig. 2C) (5.48-times respect to C group, $P < 0.05$).

3.2. Gill de novo assembly and annotation of G. chilensis

Considering that there is not a reference genome available for *G. chilensis*, and that the references transcriptomes available for this species only include liver, head kidney and muscle, we performed a *de novo* assembly of the first gill transcriptome for this species using RNA-seq libraries of twelve fish. The libraries were evaluated and filtered, obtaining after filtering a total of 613,392,496 pair-end reads (Table S2) that were used to build the gill assembly with Trinity (average pair-end reads per library of 51,116,041). The raw data of each library and experiment project is available under BioProject code PRJNA998738 at NCBI repository, with BioSamples accessions SAMN36708864, SAMN36708865, SAMN36708866, SAMN36708867, SAMN36708868, SAMN36708869, SAMN36708870, SAMN36708871, SAMN36708872, SAMN36708873, SAMN36708874 and SAMN36708875. After assembly the filtered reads a total of 58,871 supertranscripts were obtained from the Trinity assembly, with values of GC content of 48.35, and a N50 value of 2275 (Table 1), with is a commonly used metric to assess the contiguity of an assembly and represent the length of the shortest contig for which longer and equal length contigs cover at least 50 % of the assembly. Additionally, to evaluate the assembly in terms of orthologs completeness we use the BUSCO software with *Vertebrata* and *Actinopterygi* databases. The evaluation presented a 73.6% and 69.3% of complete orthologous for BUSCO score (Table S3) for *Vertebrata* and *Actinopterygi*. To identify coding potential of the assembled contigs, we detect ORFs with Transdecoder in the Trinity assembly, identifying 42,394 ORFs in the assembled contigs. Posterior to this, to reduce redundant transcripts that could be present in the transcriptome assemblies (Sayadi et al., 2016), we use CD-Hit-EST software in the Trinity assembly, obtaining 41,904 final supertranscripts.

The final assembly was annotated using BLASTx algorithm with NCBI Nr database and with functional annotation to obtain GO terms with Blast2GO software. The *de novo* gill assembly of *G. chilensis* showed a final total of 23,656 annotated transcripts. These transcripts represent a functional annotation of 55,942 GO terms (Fig. 3), of which 22,480 correspond to Biological Process (BP), 19,578 correspond to Molecular Functions (MF), and 13,884 correspond to Cellular Component (CC). In terms of the species origins of the annotations, we observed that the top-hits represented species include *Larimichthys crocea* (34.7%), *Perca flavescens* (26.9%), *Parambassis ranga* (16.4%), *Xiphophorus couchianus* (3.9%), *Gouania willdenowi* (3.2%), and *Oryzias javanicus* (3.0 %) (Fig. 4), as the top species represented in the BLAST annotation of the gill transcriptome. The *de novo* gill transcriptome assembly generated has been deposited at DDBJ/EMBL/GenBank under the accession GKNY01000000.

Table 1
Contig metrics of gill Trinity transcriptome of *G. chilensis*.

Parameter	Trinity statistics
Total trinity supertranscripts	58,871
Total trinity transcripts	192,399
Percent GC	48,35
Median contig length	1285
Average contig	1720
Contig N30	3288
Contig N40	2733
Contig N50	2275

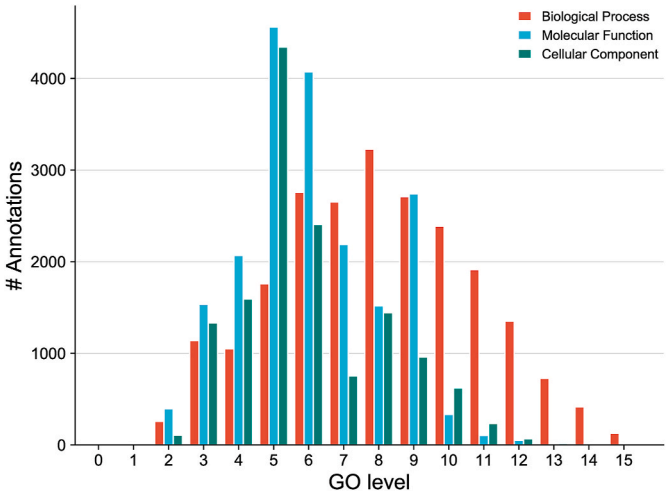


Fig. 3. Functional annotation of *de novo* gill transcriptome assembly of *Genypterus chilensis*. Number of annotations per Gene Ontology level for Biological Process (BP), Molecular Functions (MF), and Cellular Component (CC) of the *de novo* gill transcriptome assembly of *Genypterus chilensis*.

3.3. Deseq2 differential expression and GO enrichment analysis between control and stress groups in G. chilensis

To determine the effect of high temperature in the gene regulation of gills of *G. chilensis*, we performed an RNA-seq analysis of C and S groups,

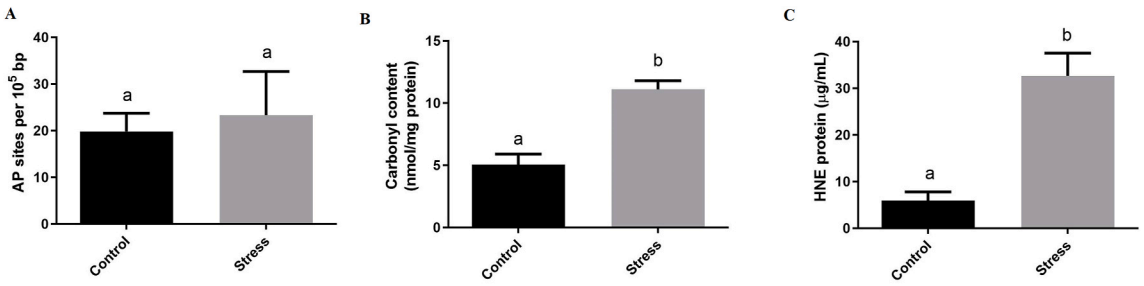


Fig. 2. Determination of oxidative damage in the gills of *Genypterus chilensis* under high temperature stress. Level of DNA damage as AP sites (A), protein carbonylation as nmol/mg protein (B), and lipoperoxidation as HNE adducts (C) in control (C) and high temperature stress (S) groups. Bars represent means ± SEM. Different letters indicate significant differences between the groups (p-value <0.05).

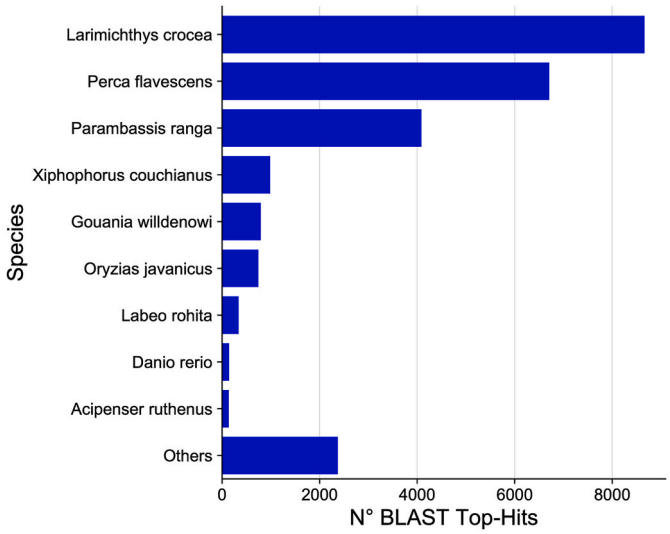


Fig. 4. Top-Hits species distribution of BLAST annotation of *de novo* gill transcriptome assembly of *Genypterus chilensis*. The graph shows the ten top species with more hits in the BLASTx annotation process of the *de novo* transcriptome assembly of the gill of *Genypterus chilensis*.

mapping the filtered reads of each library against the gill *de novo* assembly previously generated, with an average of 85.2% of mapping reads (Table S4). The unique expression values were used to generate the differential expression analysis and normalization with Deseq2, determining differential expressed transcripts between C and S groups. A total of 2669 differentially expressed transcripts (adjusted p-value of <0.01 and an absolute log2 fold change of >1) in gills between C and S due to high temperature were observed, with 1138 down-regulated transcripts (Table S5) in response to high temperature, and 1531 up-regulated transcripts (Table S6) in gills in the S group (Fig. 5A and Fig. S1). The expression patterns in the differentially expressed transcripts showed a total of four cluster of expression in gills for C and S groups (Fig. 5B).

The GO annotations of the differentially expressed transcripts were used to perform a GO enrichment analysis in response to high temperature in gills of *G. chilensis*. The analysis showed a total of four Biological Process enriched GO terms (FDR <0.05) in the down-regulated transcripts in gills under high temperature (Table 2, Fig. 6). In the up-regulated transcripts, 11 enriched GO terms (FDR <0.05) were observed (Table 3, Fig. 6), with four Biological Process, four Cellular Component and three Molecular Function enriched terms.

Using the differentially expressed transcripts, we determined the KEGG pathways most represented in response to high temperature in gills of *G. chilensis* using KO annotations. Among the most represented

Table 2
Enriched GO process represented in down-regulated transcripts in gills under high temperature in *G. chilensis*.

GO name	GO ID	GO Category	FDR
Immune response	GO:0006955	Biological Process	2.1×10^{-3}
DNA-dependent DNA replication maintenance of fidelity	GO:0045005	Biological Process	3.4×10^{-2}
Immune system process	GO:0002376	Biological Process	3.4×10^{-2}
Replication fork processing	GO:0031297	Biological Process	3.4×10^{-2}

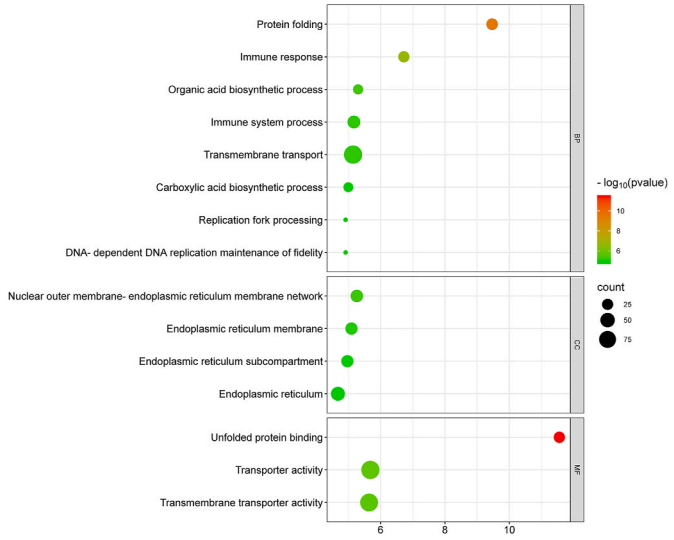


Fig. 6. Gene Ontology analysis of the enriched process in response to high temperature stress in gills of *Genypterus chilensis*. Bubble plot of the GO enriched process in the differentially expressed transcripts between C and S groups for Biological Process (BP), Molecular Functions (MF), and Cellular Component (CC). The horizontal axis represents the enrichment ratio, the dot size indicates the transcript count, and the color scale indicates $\log_{10}$ of the p-value for the enrichment analysis.

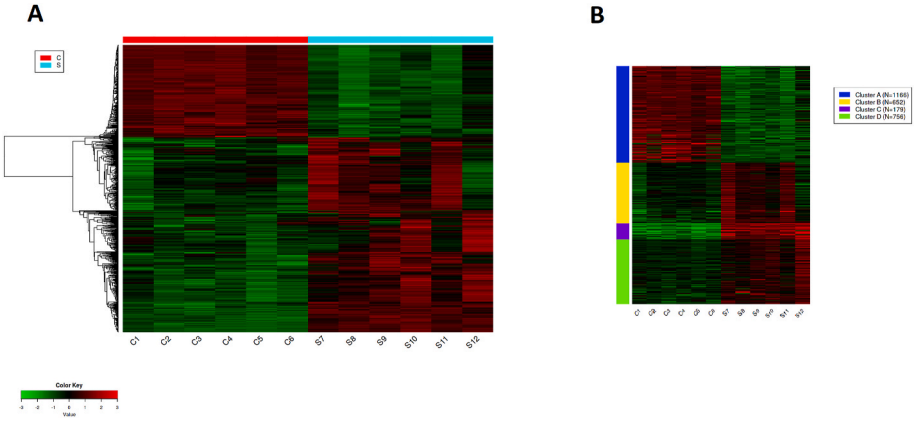


Fig. 5. Differentially expressed transcripts in response to high temperature stress in gills of *Genypterus chilensis*. The heatmap plot (A) presents the differentially expressed transcripts between control (C) and high temperature stress (S) groups. The number indicates the sample library of C and S groups. The clustering graph (B) shows the clusterization of differentially expressed transcripts between C and S groups. The letter (A, B, C, and D) indicates a different cluster, and N indicates the number of differentially expressed transcripts in each cluster.

Table 3
Enriched GO process represented in up-regulated transcripts in gills under high temperature in *G. chilensis*.

GO name	GO ID	GO Category	FDR
Protein folding	GO:0006457	Biological Process	1.9 x 10^{-6}
Organic acid biosynthetic process	GO:0016053	Biological Process	1.0 x 10^{-2}
Transmembrane transport	GO:0055085	Biological Process	1.1 x 10^{-2}
Carboxylic acid biosynthetic process	GO:0046394	Biological Process	1.2 x 10^{-2}
Nuclear outer membrane-endoplasmic reticulum membrane network	GO:0042175	Cellular Component	1.0 x 10^{-2}
Endoplasmic reticulum membrane	GO:0005789	Cellular Component	1.1 x 10^{-2}
Endoplasmic reticulum subcompartment	GO:0098827	Cellular Component	1.2 x 10^{-2}
Endoplasmic reticulum	GO:0005783	Cellular Component	2.1 x 10^{-2}
Unfolded protein binding	GO:0051082	Molecular Function	3.1 x 10^{-8}
Transporter activity	GO:0005215	Molecular Function	6.3 x 10^{-3}
Transmembrane transporter activity	GO:0022857	Molecular Function	6.3 x 10^{-3}

pathways we found pathways involved in protein processing and degradation including Protein processing in endoplasmic reticulum (04141, Fig. 7), Proteosome (03050, Fig. S2), and Ubiquitin mediated proteolysis (04120, Fig. S3), pathways associated to oxidative response and damage such as Peroxisome (04146, Fig. 8) and Base excision repair (03410, Fig. S4), pathways involved in transduction of extracellular signals including MAPK signaling pathway (04010, Fig. S5) and PI3K-Akt signaling pathway (04151, Fig. S6), and pathways associated with immune response, including Tumor Necrosis Factor (TNF) signaling pathway (04668, Fig. 9), Toll-like receptor signaling pathway (04620, Fig. S7), NOD-like receptor signaling pathway (04621, Fig. S8) and Chemokine signaling pathway (04062, Fig. S9).

3.4. Validated differentially expressed genes by qPCR

A total of fourteen differentially expressed transcripts were selected and validated through qPCR, including genes associated with proteosome (*psmd7* and *psma3*), heat shock proteins (*hsp60, hsp70,* and *hsp90)*, oxidative stress associated genes (*sod1, gst,* and *pxmp2*), and other proteins of membrane and related to several pathways (*mapk4, aqp3, aqp11, muc5ac,* and *gzma*). All the selected transcripts that present differential expression by RNA-seq, also presented significant differences by qPCR (Fig. S10, $p < 0.05$). Additionally, all the validated transcripts presented the same direction of the differences between C and S groups, with a Pearson correlation coefficient between log2FC of RNA-seq and log2FC of qPCR of $r = 0.95$ ($p < 0.0001$), confirming the differentially expressed transcripts between C and S groups in response to high temperature in gills.

4. Discussion

Poikilotherm fish experience changes in their body temperature associated with environment temperature, which could lead to change in metabolism (Sokolova, 2019), and when water temperatures increase over physiological range of the species, this could lead to a stress response in the fish (Magnadottir et al., 1999), that if is maintained in time could generate negative effect for the fish (Faught et al., 2016). This environmental stress response could variate according fish species and

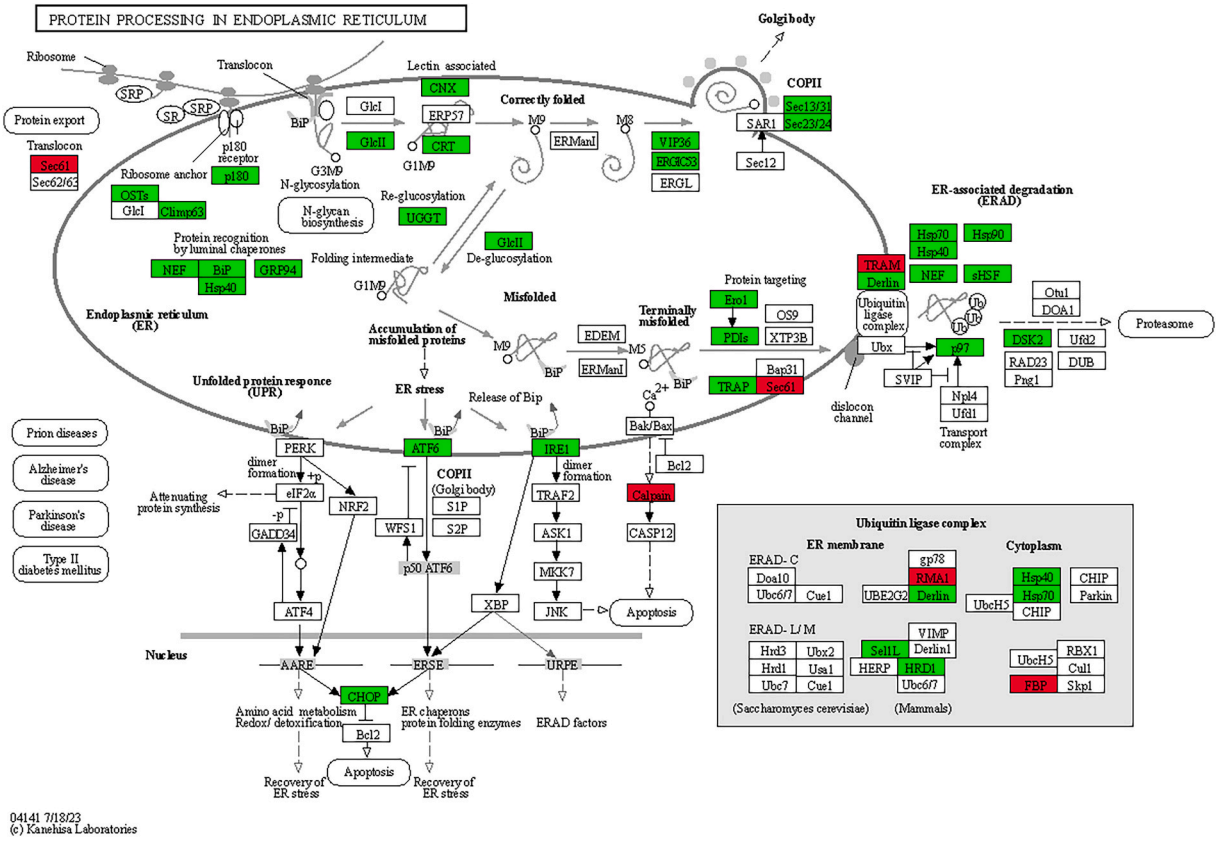


Fig. 7. Transcriptional changes in gills of *Genypterus chilensis* on response to high temperature stress on protein processing in the endoplasmic reticulum KEGG pathway. The pathway indicates on red rectangles the down-regulated transcripts and in green rectangles the up-regulated transcripts in gills between C and S groups.

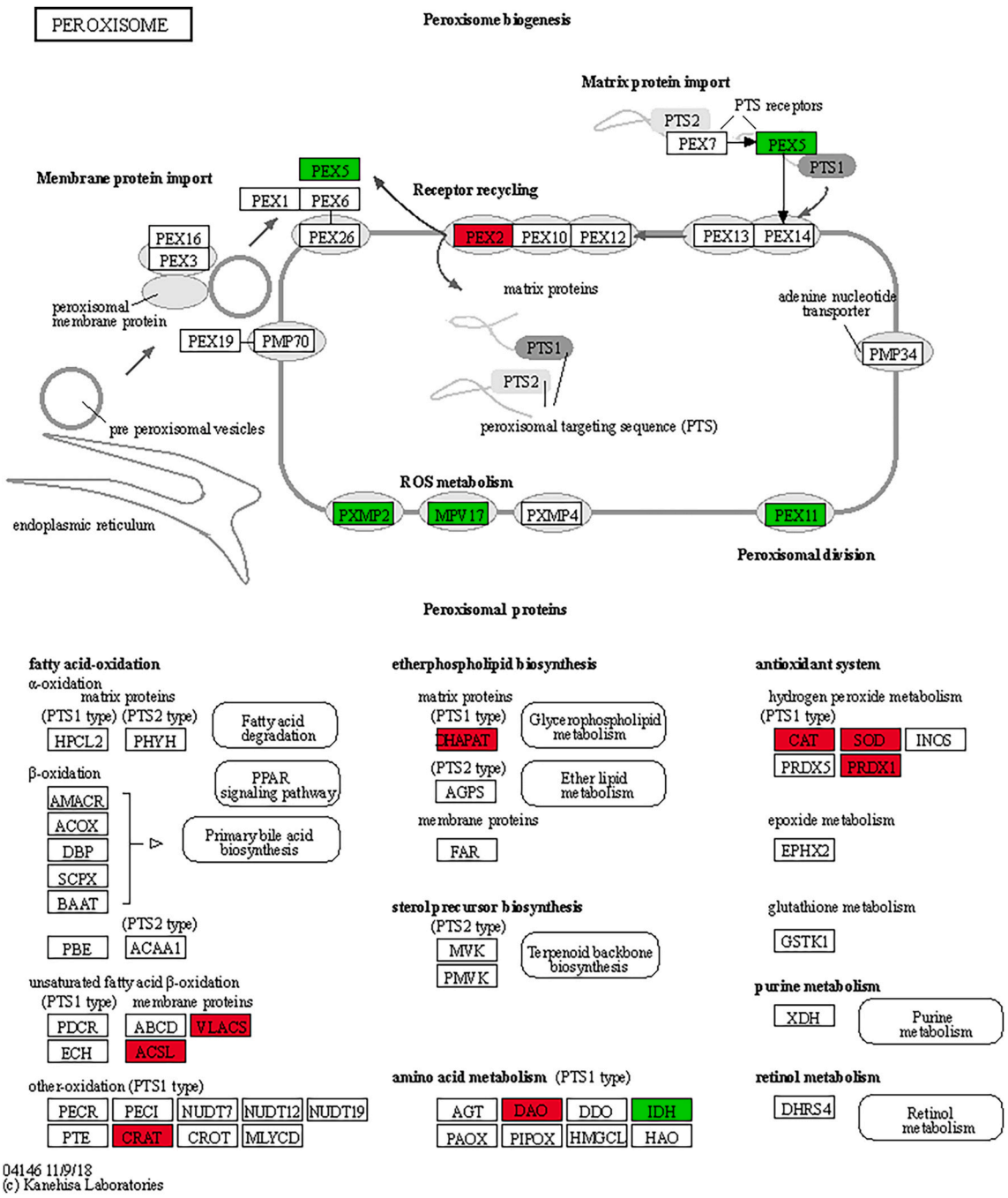


Fig. 8. Transcriptional changes in gills of *Genypterus chilensis* on response to high temperature stress on peroxisome KEGG pathway. The pathway indicates on red rectangles the down-regulated transcripts and in green rectangles the up-regulated transcripts in gills between C and S groups.

the environmental factor, as well as the specific tissue, developmental stage or cell type (Dammark et al., 2018; Eissa and Wang, 2014; Dobretsov et al., 2019; Molina et al., 2023). In *G. chilensis,* we previously studied the effect of temperature in post-ovulatory eggs and ovary, determining differences in the effect of temperature (Dettleff et al., 2021). Additionally, the transcriptional effect generated by thermal stress has been observed that could modulate different pathways and process according the tissue affected, with effect previously observed for skeletal muscle, were an induction of atrophy was generated in response to thermal stress (Dettleff et al., 2020b), while in liver an alteration of hepatic enzymes, oxidative damage, and heat shock response was observed in response to high temperature (Dettleff et al., 2022). However, in the gill, no information exists for the species, with no studies until this moment associated with gill transcriptional regulation. Additionally, in terms of genomics resources, at the moment that this study was published, no genome exists for the species, and the transcriptome references available only include skeletal muscle, head kidney and liver tissue (Aedo et al., 2014), with no sequence data in gills for the species.

In this study we generated the first the *de novo* transcriptome assembly of gills in a *Genypterus* species, with more than 23,000 annotated transcripts. The transcriptome is comparable to other the *de novo* assemblies of Chilean species as *G. chilensis* or *Seriola lalandi* (~21,000 and ~15,700 annotated sequences, respectively) (Aedo et al., 2014; Patel et al., 2016), and also to other *de novo* assemblies of gills in fish, including *Pseudopleuronectes yokohamae* (~19,000 annotated transcripts) (Cui et al., 2019), or with high annotated transcripts compared

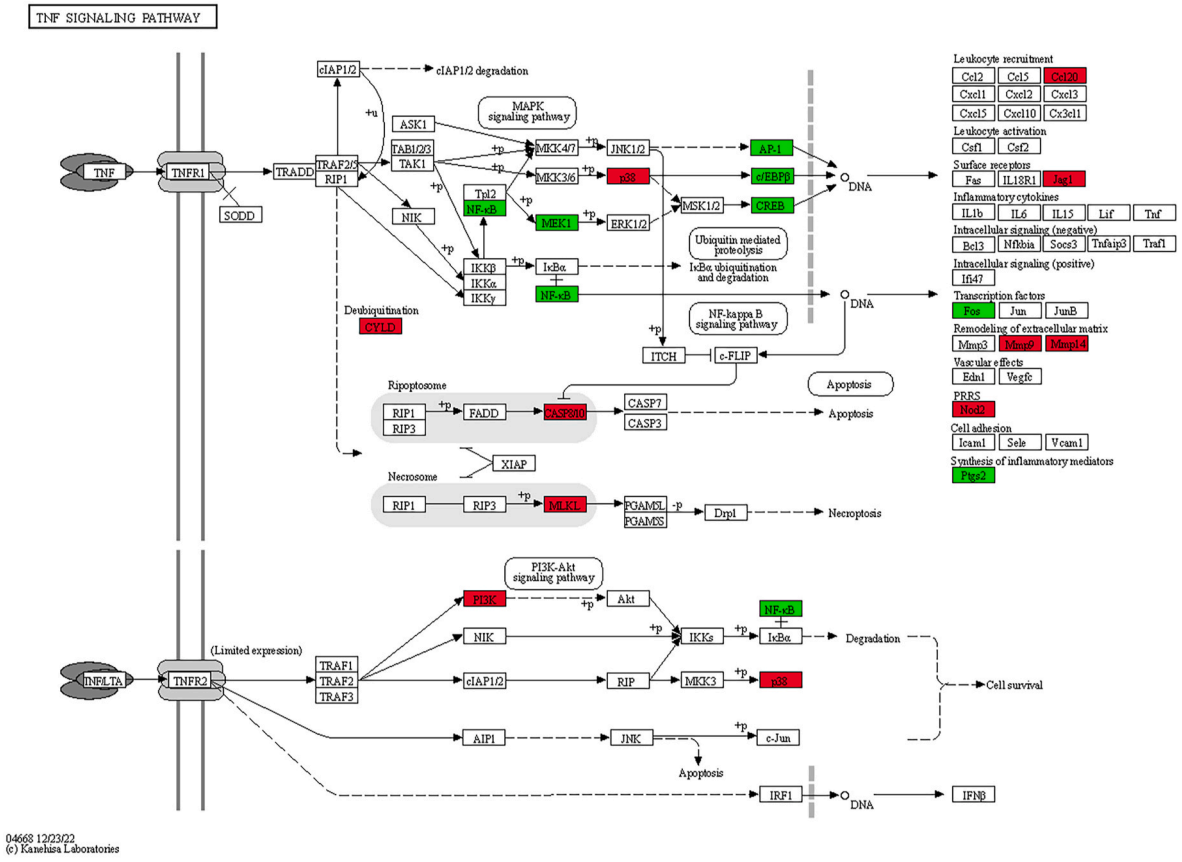


Fig. 9. Transcriptional changes in gills of *Genypterus chilensis* on response to high temperature stress on TNF signaling KEGG pathway. The pathway indicates on red rectangles the down-regulated transcripts and in green rectangles the up-regulated transcripts in gills between C and S groups.

to the gill *de novo* assembly of and *Anabas testudineus* (~13,800 annotated transcripts) (Chen et al., 2018). Also, the complete orthologous evaluation of BUSCO presented values similar to previously observed for other teleost species (~75%) (Carruthers et al., 2018), providing a valuable resource for the study of gill biology in *Genypterus* species.

4.1. Stress response and unfolded protein response in gills of G. chilensis

The stress response evaluated through cortisol increase has been previously reported for thermal stress in other teleost fish, including rainbow trout (*Oncorhynchus mykiss*) (Molina et al., 2023), goldfish (*Carassius auratus*) (Cockrem et al., 2019), Atlantic salmon (*Salmo salar*) (Vargas-Chacoff et al., 2018), among others. Additionally, gills cortisol levels has been previously used to determine stress status in teleost fish (Gesto et al., 2015). In our study, normalized gill cortisol level was significantly increased in *G. chilensis* in response to high temperature, evidencing that increases of temperatures associated to heat waves, similar to reported on Pacific coast data (SHOA, 2019) could lead to stress in *G. chilensis*. This is consistent with previous studies that has showed that marine heat waves could generate negative effects in different organisms, including plants (Bergmann et al., 2010), bivalve mollusks (De Marco et al., 2023), and fish (Mottola et al., 2020), among others, affecting physiological parameters, including cardiovascular capacity and condition index.

The stress response also can be observed at cellular level. When the cells are under a stress, an effect observed is the protein denaturation. To avoid these negative effects in the cells, prokaryotes and eukaryotes cells have a network of Hsp to prevent protein misfolding and prevent aggregates (Jacob et al., 2017). Under thermal stress, is common to observe at cellular level a Hsp response, with increases in several Hsp in response to low or high temperature stress (Metzger et al., 2016). However, the magnitude of response, as well as the specific Hsp gene responding, could present a tissue specific pattern and/or a species-specific pattern of response (Feder and Hofmann, 1999; Madeira et al., 2017). In teleost species, gill Hsp response under thermal stress has been previously observed in sockeye salmon (*Oncorhynchus nerka*) and Chinook salmon (*Oncorhynchus tshawytscha*) with *hsp90* and *serpin H1* up-regulated (Akbarzadeh et al., 2018). Also, thermal stress has showed a high up-regulation of *hsp70* in catfish (*Horabagrus brachysoma*) (Dalvi et al., 2017), *S. salar* (Vargas-Chacoff et al., 2018), and goby fish (*Gillichthys mirabilis*) (Logan and Somero, 2011), with minor up-regulation of *hsp60* in comparison with other tissues in *O. mykiss* (Shi et al., 2015).

These results are concordant with the observed in our study, with several Hsp up-regulated (with high log2FC), including *hsp10, hsp30, hsp60, hsp70, hsp90*, and *serpin H1*, with *hsp30* representing the most up-regulated transcript in the RNA-seq data (9.2 log2FC). These Hsp response under heat waves scenario observed in gills in *G. chilensis*, is concordant with the previously observed in other tissues, with increased cardiac heat shock proteins in *S. alpinus* and *S. trutta* (Mottola et al., 2020). Additionally, we have observed in previous studies that high temperature could induce Hsp in other tissues of *G. chilensis*, with increase of *hsp60* and *hsp70* in skeletal muscle (Dettleff et al., 2020b), increase in *hsp60* in post-ovulatory eggs, with no effect in ovary (Dettleff et al., 2021), and *hsp40, hsp60, hsp70, hsp90,* and *serphin H1* up-regulation in liver under thermal stress (Dettleff et al., 2022), showing a general Hsp response to thermal stress, but with a tissue specific pattern of response.

Additionally, an unfolded protein response was observed in gills under high temperature in *G. chilensis*. The unfolded protein response is a mechanism to maintain an optimal rate of protein production, and is activated when a endoplasmic reticulum (ER) stress is generated,

product disruption on normal protein folding on ER, with generation of unfolded or misfolded proteins (Hetz et al., 2020). This unfolded protein response in fish has been observed in Tambaqui (*Colossoma macropomum*) (Fe-Goncalves et al., 2020), Nile tilapia (*Oreochromis niloticus*) (Li et al., 2021), and *S. aurata* (Mininni et al., 2014) under thermal stress. This is consistent with our results, were several processes associated were enriched, including protein folding (BP), endoplasmic reticulum (CC), and unfolded protein binding (MF), as well as the differentially expression of several transcript of the KEGG pathways protein processing in endoplasmic reticulum. This unfolded protein response also has been previously observed in liver of *G. chilensis* under thermal stress (Dettleff et al., 2022), evidencing that increase in temperature could affect the normal folding of proteins in gills and other tissues.

4.2. High temperature generates oxidative stress in gills

At cellular level, under a condition of stress, a disturbance of oxidative status could be generated (Letavayova et al., 2006), producing oxidative stress due to environmental stressor in teleost fish (Madeira et al., 2013; Vinagre et al., 2012). In cells, the oxidative status of cells is mediated by the balance between oxidant agent, including reactive oxygen species (ROS), and antioxidant agents, including antioxidant enzymes (Betteridge, 2000). These ROS play key function in cells, participating in signal transductions, however, also could affect the molecules of the cells, inducing oxidative damage in DNA, proteins and lipids (Lesser, 2006). In fish, high temperature generates higher respiratory rates, hypoxia and stress status, which increases the ROS production (Lesser, 2006; Madeira et al., 2016). The temperature could induce oxidative damage in several tissues in fish (Cheng et al., 2018; Bacchetta et al., 2020). The external organs such as gills are directly exposed to environmental stressors such as temperature, which could lead to oxidative stress in this tissue (Lesser, 2006). In our study, which is the first in study the oxidative status in gills under environmental stressors in *Genypterus* species, oxidative damage was observed at protein carbonylation and lipoperoxidation in gills, but with no damage at DNA level. This oxidative damage effect of temperature has been previously observed in gills of *Gobius paganellus* (Vinagre et al., 2014) and *Etroplus suratensis* (Joy et al., 2017), with a significant increase of lipoperoxidation in these species. This effect of temperature also has been observed in combination with hypoxia in pacu (*Piaractus mesopotamicus*) associated to lipoperoxidation in gills (Nitz et al., 2020). However, in other fish species such the Antarctic fish *Notothenia coriiceps*, increased temperature by 6 days do not induce oxidative damage in gills or muscle, with damage observed as lipoperoxidation in brain and liver of this species (Klein et al., 2017). This effect also has been observed in the fish *Notothenia rossii,* were increased temperature generate lipoperoxidation and protein carbonylation in liver, but no in gills or brain (Guillen et al., 2022), evidencing that the effect of temperature to generate oxidative damage could variate according to tissue in the same species. In *G. chilensis* this phenomena has been previously observed, with thermal stress generating oxidative damage in muscle (Dettleff et al., 2020b) and liver (Dettleff et al., 2022), but with no oxidative damage effect on ovary of this species (Dettleff et al., 2021).

The oxidative status observed by damage markers in gills in our study was also consistent with the observed in the RNA-seq data, were several transcripts associated to peroxisome pathway were differentially expressed, including a down-regulation of several transcripts associated to antioxidant system (*sod, cat, prdx1*), and the up-regulation of several peroxisome associated proteins (*pex5, pex11, pxmp2, mpv17*). Under oxidative stress, cells use the antioxidant system to reduce ROS levels, however, this system could be overwhelmed, leading to oxidative damage (Teles et al., 2019). The regulation of antioxidant enzymes under thermal stress in fish has been previously observed, with up-regulation of these genes in several fish and tissues, including black porgy (*Acanthopagrus schlegeli*) (An et al., 2010), pufferfish (*Takifugu obscurus*) (Cheng et al., 2015), European sea bass (*Dicentrarchus labrax*) and milk fish (*Chanos chanos*)(Chang et al., 2021). However, also has been previously down-regulation of antioxidant enzymes in response to high temperature in some fish species, including a decrease in *gpx* in liver of *Solea senegalensis* (Castro et al., 2012). The peroxisome is an organelle with several functions, including fatty acid β-oxidation, synthesis of ether glycerolipid, and redox homeostasis (De Duve and Baudhuin, 1966), containing antioxidant enzymes and ROS-producing enzymes, playing also a role in the ROS metabolism (Okumoto et al., 2020). In fish, previous studies has shown a regulation of peroxisome associated genes in response to environmental stressors, including pollution with organic contaminants (Olivares-Rubio and Vega-López, 2016; Cajaraville et al., 2003). In zebrafish (*Danio rerio*) has been show that peroxisome play a relevant role in stress response under oxidative stress condition (Wang et al., 2022), as well as in the Antarctic fish *Trematomus bernacchii* were peroxiredoxin are differential expressed in response to temperature (Tolomeo et al., 2019), which could be consistent with the observed in the *G. chilensis* in gills under thermal stress.

4.3. Immune modulation in response to high temperature in G. chilensis

The immune response in fish has evolved to face infections by external agents that could affect the health of the fish, generating an initial innate immune response and a posterior adaptative immune response to face these challenges (Thompson, 2017). In this study, we evaluate by first time the transcriptional effect of high temperature on gill transcriptomic of a *Genypterus* species trough RNA-seq, allowing determine the effect on immune related genes in this tissue. A modulation of immune response was observed in gills of *G. chilensis* due to thermal stress, with an enrichment of immune response (BP) and immune system process (BP) terms in the down-regulated genes, evidencing a relevant regulation of the immune process in this tissue. These results are relevant, considering that gills represent a direct point of entry of pathogens considering that act as a direct interphase with aquatic environment, and play a relevant role in the immune response in fish (Koppang et al., 2015). This immune response is perform by GIALT, through the activity of leukocytes, cytokines and other active molecules (Resseguier et al., 2020). Effect of temperature on immune response has been previously reported on mammals, with a decrease on immune genes, including several cytokines (interleukins, interferon γ, and TNF-α) (Bagath et al., 2019), with an inhibition of pro-inflammatory cytokines (Chauhan et al., 2021). In fish, temperature generate an important modulation of the innate immune system, including cellular response, complement system and pro-inflammatory response, as well as on adaptative immune system, including lymphocytes T and B, as previously observed for several teleost species under low temperature stress (Abram et al., 2017). In some species, the effect of high temperature has showed an increase of immune related genes, such as Atlantic cod (*Gadus morhua*) with increased humoral immune response (Magnadottir et al., 1999), while in other species an immunomodulation was observed, with a down-regulation of several immune related genes in spleen of grass carp (*Ctenopharyngodon idellus*) using RNA-seq (Yang et al., 2016). Similarly, the observed on the previous study, we observed down-regulated transcripts associated to complement system (cf*1*, *c7, ctrp1, ctrp5, ctrp9*), inflammatory cytokines (*il12b, ifng*), as well as differential regulation of Toll-like receptor signaling pathway, and TNF signaling pathway. Differential regulation on immune genes has been previously observed in gills of *O. niloticus*, with down-regulation of complement genes as *c3,* and up-regulation of *nfkb,* like our results, with also an up-regulation of *tnfa* (Esam et al., 2022). In our case, not differentially expression was observed for *tnfa* transcript, but the TNF signaling pathway and *tnfa* receptors were differentially expressed in respond to thermal stress. The TNFα mediate inflammatory response and innate immunity, activating a cytokine-mediated cellular response (Janes et al., 2006). TNFα signaling also activate the MAP kinase

signaling pathways, that transduce a variety of extracellular signals that regulate cellular responses, including the stress-activated MAP kinases p38 and JNK (Sabio and Davis, 2014). This regulation of MAP kinase signaling pathways by the TNF signaling pathway is consistent with our results, were MAP kinase signaling pathways was differentially regulated, including the down-regulation of *p38* transcript. The differential regulation in gills of *G. chilensis* of immune pathways with a predominant down-regulation in Toll-like receptor, NOD-like receptor and chemokine signaling pathways evidence the effect of thermal stress in the regulation of immune related pathways. However, the variable response observed for some immune transcripts in terms of up and down regulation also was observed previously in response to thermal stress in other teleost fish in spleen of *C. idellus* (Yang et al., 2016), gills of *O. niloticus* (Esam et al., 2022), spleen and gills of *O. mykiss* (Guo et al., 2023), and marine invertebrates (Li et al., 2019; Jiang et al., 2018; Dettleff et al., 2020a), evidencing the complexity of the immune response and thermal stress in marine organisms. In the context of climate change, impact of heat waves in gills immune capacity could be relevant, requiring more study to deeply understand the effect of climate change on host-pathogen interaction and capacity of *Genypterus* species to face pathogens under high temperature scenario.

5. Conclusion

The present study evaluated the effect of thermal stress in gills of *G. chilensis* by the first time, using a *de novo* assembly approach to identify the effect of simulated heat waves on transcriptomic and oxidative stress status. We showed that gills could be affected by thermal stress, allowing its evaluation through direct measurement of gill cortisol, with negative effect in gills, including oxidative damage, as well as and unfolded protein response, and immune modulation in gills of this species. These results should be considered under a climate change scenario, especially in the short-term effects for aquaculture species, such as heat waves, that could impact biology of the fish.

Availability of data set

The sequencing data generated for this study is available as raw data under BioProject code PRJNA998738 at NCBI repository (BioSamples accessions SAMN36708864, SAMN36708865, SAMN36708866, SAMN36708867, SAMN36708868, SAMN36708869, SAMN36708870, SAMN36708871, SAMN36708872, SAMN36708873, SAMN36708874 and SAMN36708875). The *de novo* gill transcriptome assembly generated his available at DDBJ/EMBL/GenBank under the accession GKNY01000000.

Animal and human rights statement

This study and all the procedures performed with black cusk-eel were approved by the bioethical committee of Andres Bello University (number 007/2018) following animal welfare procedures and guidelines of the National Research and Development Agency (ANID) of the Chilean government.

CRediT authorship contribution statement

Phillip Dettleff: Writing – original draft, Visualization, Validation, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Carla Toloza:** Validation, Methodology, Formal analysis. **Marcia Fuentes:** Validation, Methodology, Conceptualization. **Jorge Aedo:** Methodology, Investigation. **Rodrigo Zuloaga:** Validation, Methodology, Formal analysis. **Juan Manuel Estrada:** Methodology, Investigation. **Alfredo Molina:** Resources, Project administration, Investigation, Funding acquisition. **Juan Antonio Valdés:** Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Phillip Dettleff reports equipment, drugs, or supplies and travel were provided by National Agency for Research and Development. Juan Antonio Valdes reports equipment, drugs, or supplies and travel were provided by National Agency for Research and Development. Alfredo Molina reports equipment, drugs, or supplies was provided by National Agency for Research and Development. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marenvres.2024.106440.

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