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Molecular response of a sub-Antarctic population of the blue mussel (*Mytilus edulis platensis*) to a moderate thermal stress.

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Abstract

The Kerguelen Islands (49°26'S, 69°50'E) represent a unique environment due to their geographical isolation, which protects them from anthropogenic pollution. The ability of the endemic mussel, part of the *Mytilus* complex, to cope with moderate heat stress was explored using omic tools. Transcripts involved in six major metabolic functions were selected and the qRT-PCR data indicated mainly changes in aerobic and anaerobic energy metabolism and stress response. Proteomic comparisons revealed a typical stress response pattern with cytoskeleton modifications and elements suggesting increased energy metabolism. Results also suggest conservation of protein homeostasis by the long-lasting presence of HSP while a general decrease in transcription is observed. The overall findings are consistent with an adaptive response to moderate stresses in mussels in good physiological condition, i.e. living in a low-impact site, and with the literature concerning this model species. Therefore, local blue mussels could be advantageously integrated into biomonitoring strategies, especially in the context of Global Change.

31

32 **Key words**

33 Kerguelen island, *Mytilus* sp., indicator species, gills, abiotic stress, temperature, qRT-PCR, 2DE,

34 biomonitoring

35

1. Introduction

The sub-Antarctic islands are amongst the most isolated territories from any continental landmass and contain a number of marine, coastal habitats present at these latitudes. On one hand, due to rapid and drastic changes in climatic conditions at high latitudes, accurate assessments of the sensitivity and vulnerability of polar organisms must be achieved in order to reliably predict possible changes in those ecosystems. On the other hand, thanks to scientific projects conducted in this location for decades, observation and experimentation on sentinel species provide priceless opportunities to improve our knowledge of adaptive responses and ecophysiological strategies in invertebrates from naturally, highly variable environments, such as intertidal habitats (Caza et al., 2015; Suda et al., 2015; Guillaumot et al., 2020). In addition, the physicochemical quality of marine waters is much influenced by both hydrologic factors and atmospheric inputs. Hence, these isolated marine ecosystems have been found to face anthropogenic pressure, as trace contaminations were reported in aquatic habitats and biota (Carravieri et al., 2013; Jaffal et al., 2015).

Scientific consensus has been reached on Global Change and several scenarios predict significant average temperature increase toward 2100 (Intergovernmental Panel on Climate Change-IPCC, Pachauri et al., 2014; Pörtner et al., 2019). They anticipate not only a global warming, but also an increase of extreme events such as heat waves. One challenge we face is to decipher temperature impact on organisms when combined with pre-existing stress. Marine invertebrates have been widely used as bioindicators of coastal water quality, mainly for monitoring chemical pollution, in order to measure site contamination levels but also to assess biological effects on invertebrate communities (Auffret, 2005; Beyer et al., 2017). The intertidal belt of the Kerguelen archipelago is inhabited by numerous mussel beds. Two species share the same rocky shore habitat: the Antarctic sub-species of blue mussel, *Mytilus edulis platensis* (*M. edulis platensis*) and the ribbed mussel *Aulacomya atra*. Blue mussels are keystone species in coastal ecosystems distributed in many oceans as a result of genetic differentiation within smooth-shelled mussels, now grouped in the so-called *Mytilus* complex. In the Northern Hemisphere, the *M. edulis* complex of species is composed of three genetically well delineated taxa: *M. edulis*, *M. galloprovincialis* and *M. trossulus* (Gerard et al, 2008). The occurrence of blue mussels has also been reported in the Southern Hemisphere, as *M. galloprovincialis* and so-called "native Chilean smooth-shelled *Mytilus*" at the southern tip of the South American continent (Borsa et al, 2012). The latter species was assigned at a subspecific rank and named *M. edulis platensis* by these

66 authors. Kerguelen mussels, earlier described as *M. desolationis*, are now assigned as *M. edulis*
67 *platensis* (Gerard et al, 2015). To our knowledge, there is no report relating the occurrence of other
68 members of the genus *Mytilus* at the Kerguelen archipelago, nor was any introduction reported to date.
69 The ecophysiological status of mussel populations remains largely unknown. In a recent study, Caza et
70 al. (2015) compared the immune status of the two cohabiting mussel species and observed that
71 responses to experimental abiotic changes differed between these species suggesting possible
72 contrasted resilience to environmental stress.

73 The present study focused on stress ecophysiology of the endemic blue mussels collected from natural
74 populations in response to temperature stress. Investigations in those osmo- and thermo-conformers
75 were expected to produce valuable baseline biomarker signatures that would be relevant for monitoring
76 both anthropogenic and climatic pressure on high latitudes and other pristine coastal ecosystems.
77 Temperature variations were found to modify physiological processes such as respiration and nutrition,
78 but also numerous molecular pathways, with species-specific thermal tolerance (Kültz, 2020; Somero,
79 2020). At the physiological level, small temperature variation increases respiration and nutrition
80 processes, but when more stressful temperature occurs, metabolism shuts down with marked decrease
81 of respiration rate.

82 In bivalve mollusks, respiration and nutrition are supported by the gills, an interface organ between the
83 mussel and its environment. In the present work, gill tissues collected from mussels exposed to a mild
84 thermal stress were analyzed by proteomic and transcriptomic tools using two-dimensional gel
85 electrophoresis (2DE) and qPCR, respectively. On-gel 2DE constitutes an open approach to analyze
86 complex proteomic patterns and detect differentially abundant proteoforms (DAPs) (Rabilloud et al.,
87 2010; Lee et al., 2020). In parallel with the proteomic analyses, the mRNA expression of 51 genes was
88 monitored to determine the impact of exposure to increased temperature on the expression pattern of
89 individuals. These genes were chosen to cover different metabolic pathways (energetic metabolism,
90 oxidative stress scavenging, stress response, detoxication, immune response and cell cycle regulation),
91 on the basis of previous studies. Hence, molecular components involved in the process of adaptation
92 and resistance to thermal stress are numerous and have been reviewed in several publications of
93 interest (for examples, please see: Somero, 2010; Lockwood and Somero, 2011; Fields et al., 2012;
94 Tomanek, 2012, 2014; Péden et al., 2016; Clark et al., 2017; Coppola et al., 2018; Feidantsis et al.,
95 2020). In addition, we genotyped individuals (Glu-5' locus, Inoue et al.; 1995; Gerard et al., 2015) to

verify whether there is potential inter-individual variability in the response pattern to temperature change. In summary, this study aimed to investigate the ability of the endemic blue mussels to cope with a thermal stress.

2. Material and Methods

2.1. Site description and mussel collection

Kerguelen is a small, isolated archipelago in the Southern Indian Ocean characterized by a highly heterogeneous environment. The proximity of the polar front generates a temperature contrast between the northern and the southern coasts. The circumpolar, oceanic front creates two separated branches from the western side of the main island. Combined with high freshwater inputs from many rivers, waters in the northeastern part of the archipelago have relatively lower salinity, as in most locations at the mouth of many small coastal rivers.

Seawater temperatures at Kerguelen were obtained from the long-term monitoring program "Proteker" (Féral et al. 2016). It revealed that between 2012 and 2019 the mean annual temperature in the Baie du Morbihan where mussels were collected varied between 4.1 and 5.5°C at 5 m depth. The maximums observed ranged between 5.6 and 11.1°C during the austral summer while the minimums ranged between 1.0 and 4.6°C in the austral winter (<http://www.proteker.net/-Thermorecorders-.html>).

Mussels (*M. edulis platensis*) (65.23 ± 4.55 mm shell length) were collected from Kerguelen Archipelago (Bras-Laboureur: 049°27.897S / 069°66.086E) in December of 2013. The mussels were sampled at once in the intertidal zone and kept in 20L containers filled with clean, aerated seawater collected offshore at the sampling site. The water was oxygenated by aeration using a battery-operated portable air pump and organisms were maintained throughout the transport phase at a water temperature close to those measured on field (7.5°C).

Once at the Port-aux-Français laboratory (approximately 6h post-harvest), the mussels were divided in homogenous groups of 50 individuals per 50L aerated aquarium tanks containing filtered recirculating seawater maintained on a 12h:12h light cycle (Fig. 1). A 7-days acclimation period was conducted at 7.5°C, which corresponds to the water temperature observed in the field. A first sampling was then performed for gene expression analysis (T0-control and T0-test). After acclimation, one group of mussels was maintained at the field temperature while the other group was exposed to a mild thermal challenge. Specifically, water temperature was increased from 7.5 to 20°C over a period of 7 days

(+1.8°C per day). Finally, mussels were kept under their thermal conditions with daily follow up of temperature, salinity and pH until their sacrifice at T28 (group T28-control and group T28-test). For all sampling, gills were dissected out, immediately frozen in liquid nitrogen and stored at -80°C until analysis. Our experimental design is based on the individual as the sampling unit (and thus, as the replication level), and the level at which we want to assess variations in gene expression and proteomics.

2.2. Gene expression

2.2.1. RNA extraction and qRT-PCR analysis

Total RNA was extracted from gill tissue using TRI Reagent® solution (Ambion) according to the manufacturer's instructions (n=10 per condition). Three µg of total RNA were reverse transcribed using M-MLV reverse transcriptase (Promega, Madison, WI), random hexamers (Promega) and an anchor-oligo(dT) primer (5'-CGCTCTAGAACTAGTGGATCT₍₁₇₎-3'). The relative mRNA expression of 51 genes selected for their involvement in different metabolisms was estimated by real-time PCR amplification using specific primers (Table S1). A volume of 2.1 µl of each diluted reverse transcription product (1:100) was subjected to real-time PCR in a final volume of 5 µl containing 0.4µM of primers and 1X LightCycler® 480 SYBR green I master (Roche, Germany). The amplification was carried out as follows: initial enzyme activation at 95°C for 6 min, then 40 cycles of 95°C for 10 sec, hybridization at 60°C for 30 sec and elongation at 72°C for 30 sec. Calibration curves were obtained using serial dilution of a cDNA pool from 12 individuals. In this study, we used a fragment of 28S as a reference gene, because other commonly used reference genes (such as EF1α or actin) are often found regulated in stressed bivalves (Boutet et al., 2004; Tanguy et al., 2004; David et al., 2005). Relative expression of each gene was calculated according to comparative Ct method using the formula: $RQ = 2^{-\Delta Ct}$.

2.2.2. Statistical analysis

In order to assess modifications of transcriptomics response patterns according to time and temperature, all the dataset was analyzed by using a PLS-DA (Partial Least Square Discriminant Analysis, MixOmics package version 6.6.2., Le Cao et al. 2016). This method is appropriate for experimental designs with far more variables than individuals within each group, and when the dataset contains missing values. It allows identification of the gene expression levels that are the most suitable to separate exposure conditions, *i.e.* whose variations are characteristics of each condition.

Important genes were selected based on the VIP (Variable Importance in the Projection) values, with a threshold value of 1. Exposure conditions were then plotted on a factorial plane combining the two first axes of the PLS-DA, while variables were plotted on a correlation circle, giving the correlation of each variable with the axes of the PLS-DA. The significance of the exposure condition discrimination was assessed by a permutation test based on a cross-validation test (Westerhuis et al., 2010; Szymańska et al., 2012, RVAideMemoire package version 0.9-75). Here, the structure described by our classification variable (exposure condition) was analyzed using a “null model”. In this model, gene expression data are randomized among the two groups, to generate a structure where chance is the only driver of the dataset. At each iteration, the observed and the random structure are compared, and the model is significant if the discrimination is better with the observed rather than with the random dataset. In a first step, the test was performed to define whether the built model was significant, hence that at least one group is significantly discriminated from the others. Once this first assessment done, a second test performs paired comparisons among the four experimental groups with an FDR (false detection rate) adjustment of p-values, to further explore gene expression differences between each group. All analyses were performed with RStudio version 1.1.463, with R version 3.6.3 (R Core Team 2000, RStudio Team 2016).

2.2.3. *Glu-5'* genotyping

After extracting DNA from the gills using a standard phenol-chloroform-isoamyl alcohol extraction protocol (25:24:1), the locus *Glu-5'* was amplified with primers Me-15 (5'-CCAGTATACAAACCTGTGAAGAC-3') and Me-17 (5'-CTGGTGGATAATTTGTCTTTGC-3'). This locus is located at the 5' extremity of exon Glu coding for an adhesive foot protein (Inoue et al. 1995; Rawson et al., 1996). This locus contains an insertion/deletion (indel) zone, whose amplification reveals three alleles: (T, E and G) that, respectively distinguish *M. trossulus*, *M. edulis*, and *M. galloprovincialis* in the Northern Hemisphere (Borsa et al., 1999). Here, the amplification of the *Glu-5'* exon produced 210-bp (allele E) and 160-bp (allele G) fragments, typical of, respectively, *M. edulis* and *M. galloprovincialis* from the Northern Hemisphere (Inoue et al., 1995), but allowing identification of local adaptations of *M. edulis platensis* in Kerguelen (Gerard et al, 2015). The genotypes at *Glu-5'* were determined from fragment-length variation on 2% agarose gels.

Differences in gene expression according to the genotype was studied through a PCA. Since the database contains missing values, a first step was to impute missing values using the imputePCA function of the missMDA package v1.18 (Josse and Huchon, 2013, 2016). Then a classical PCA was performed with the ade4 package v1.7-15 (Dray and Dufour, 2007). To confirm the absence of structure observed in the factorial plane, a MANOVA on the individuals coordinates on the two first axes of the PCA, with the exposure condition x genotype combination as independent variable was performed. Since the experimental design was not equilibrated for genotype, since it's only a posteriori data, no systematic test was possible to compare gene expression at each condition and to test for interaction between exposure duration and temperature. However, some control can be performed. The tank effect at T0 was compared with the (EG) genotype. After checking for normality and homoscedasticity, we performed either a student t test or a Welch t test. With the same procedure, we tested the exposure duration effect on gene expression with the (EE) genotype for the control tank (n=3 for T0, n=4 for T28). Finally, the genotype effect was assessed at T0, in the test tank (n=3 for (EE) genotype and n=6 for (EG) genotype).

2.3. Proteome analysis and statistical treatment

Protein extraction and two-dimensional analysis were performed as previously described in Péden et al., 2016. Gills of mussels sacrificed at day 28 (two conditions: group T28-control and group T28-test, n=8) were ground using Precellys® Homogenizers and sonicated in a 1:4 ratio of lysis buffer (9 M urea, 2% CHAPS, 65 mM dithioerythritol, 0.02% pharmalyte 3-10NL, GE Healthcare and 16 µg·mL⁻¹ aprotinin). Cellular debris was removed by centrifugation at 10000x g for 20 min at 20°C and protein concentrations of supernatants were determined according to the method of Bradford with bovine serum albumin as a standard. For 2D electrophoresis, 750 µg of proteins per sample were loaded on non-linear wide-range immobilized pH gradients (IPG strip pH 3-10, NL/18 cm, GE Healthcare). The IEF (isoelectrofocusing) was carried out with a horizontal electrophoresis apparatus (Multiphor, Amersham Pharmacia Biotech). The electric parameters were as follow: 500 V for 0.01 h (gradient), 500 V for 5 h, 3500 V for 5 h (gradient) and 3500 V for 9.5 h. After IEF, strips were incubated for 15 min in an equilibration buffer (0.5 M Tris buffer pH 6.8 with 6 M urea, 1% SDS, 26% glycerol) containing first, 30 mM dithiothreitol and second, 245 mM iodoacetamide. SDS-PAGE electrophoresis was performed with a Protean plus Dodeca-Cell (Bio-Rad) allowing a parallel run of 12 large gels (12% polyacrylamide, 20

cm x 20 cm x 1.5 cm). After migration, proteins were visualized by colloidal blue staining. Gels were scanned using the GS-800™ Calibrated Densitometer (Bio-Rad) and exported from Quantity One as raw file (16-bits, .tif). Only gels exhibiting highly reproducible patterns were selected for analysis (4 per condition). Images were analyzed using Delta 2D (Decodon, GmbH). With this software, a single composite image named the fusion gel was created using the group warping strategy to connect gel images through match vectors. After quality checking, a gel fusion of 957 spots was obtained. The spot boundaries detected on the fusion gel were subsequently transferred back to all the gels, establishing spot parameters. For each spot, the normalized volume was expressed as percentage of the total volume of all spots of the gel. Normalized volumes were compared between control group (group T28-control) and group of mussels exposed to 20°C (group T28-test) following a Student t-test. A total of 61 spots passed the $p < 0.01$ cut-off and of these, 49 were determined to be correctly delimited on all individual gels and therefore were considered as significantly different.

2.4. MS/MS and protein identification

Spots of interest were excised manually from colloidal blue stained 2D gel and submitted to in gel digest by trypsin (Promega France). After digestion, peptides were dried on a speedvac, resuspended in 15 µL of 3% (v/v) acetonitrile and 0.1% (v/v) formic acid, and then analyzed with a nano-LC1200 system coupled to a Q-TOF 6520 mass spectrometer equipped with a nanospray source and an HPLC-chip cube interface (Agilent Technologies). A 30-min linear gradient (3-80% acetonitrile in 0.1% formic acid), at a flow rate of 370 nL/min, was used to separate peptides on polaris-HR-Chip C18 column (150 mm long x 75 µm inner diameter). Full autoMS1 scans from 200 to 1700 m/z and autoMS2 from 9 to 1700 m/z were recorded. In every cycle, a maximum of 5 precursors sort by charge state (2+ referred and single-charged ions excluded) were isolated and fragmented in the collision cell. Collision cell energy was automatically adjusted depending on the m/z. Active exclusion of these precursors was enabled after 2 spectra within 1 min, and the threshold for precursor selection was set to 4000 counts. A peak list for database searching was created by using Masshunter Workstation-Qualitative Analysis (version B.04.00; Agilent Technologies). For protein identification, MS/MS peak lists were extracted and analyzed with X!tandem pipeline (<http://pappso.inrae.fr/>). Mass spectra were investigated using protein databanks translated with TransDecoder from RNA-seq data of *Mytilus galloprovincialis* and *Crassostrea gigas* species (Moreira et al., 2015). The searches were performed with the following specific parameters:

enzyme specificity, trypsin; one missed cleavage permitted; variable modifications, methionine oxidation, cysteine carbamidomethylation; peptide mass tolerance, 10 ppm; fragment mass tolerance, 0.5 Da. As filter identification results, the following parameters were used: peptide Evalue, 0.05; minimum number of peptides per protein, 2; protein Evalue -4.0. Protein blast search was done against the non-redundant protein database from NCBI (<https://blast.ncbi.nlm.nih.gov>).

2.6. Protein network analysis

Association network analysis of proteins was conducted using STRING (<https://string-db.org>; Szklarczyk et al., 2019). STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) is a database of known and predicted protein-protein interactions, including physical and functional associations. The data stem from computational prediction, from knowledge transfer between organisms, and from interactions aggregated from other databases. The interaction map was generated from the web-based search STRING v11.0 database (<https://string-db.org/>). Default settings are as follows: evidences were selected for meaning of network edges; all active interaction sources were used for linkage; minimum required interaction score was fixed at medium confidence (0.400). Network nodes represent proteins. Edges represent protein-protein associations, meant to be specific and meaningful, *i.e.* proteins jointly contribute to a shared function.

3. Results & discussion

The average temperature at Kerguelen depends on numerous factors, including depth, localization, seasons, currents, etc. In summer, the typical temperature at a 5m depth is approximately 7-8°C (Féral et al., 2016). In summer, during low tide, however, the temperature in mussel beds can reach more than 10-15°C, and possibly more in sunny conditions. The 20°C exposure condition is thus not an unrealistic scenario and is most likely to become a common event given the increase of seawater temperature at Kerguelen. The 28-day period is an experimental condition that more or less represents a semi-chronic exposure often used to study the effect of specific factors on the health status of mussels (Beaudry et al., 2016; Coppola et al., 2018; Lenz et al., 2018, etc.).

A recurrent debate exists within scientific literature on what is the appropriate level of replication in ecological and ecotoxicological studies (Bastos et al., 2013; Devin et al., 2014; Davies and Gray, 2015). Indeed, pseudoreplication can't be avoided, and a tank effect can have occurred in our experiment.

However, given the tested hypothesis and the recorded biological responses, it's far more likely that it's the thermal stress that induced such effects.

3.1. Transcriptional analysis reveals metabolic adaptations

Our results indicate that mRNA expression profiles are significantly different between groups of mussels subjected or not to a temperature increase (Table 1 and Table S2 for significant and non-significant results, respectively). The gene expression levels allow a better discrimination than a null model, with a classification error rate of 0.56 ($p=0.008$). Such a high level of error rate is not surprising, since no differences were expected between the two T0 tanks (T0-control and T0-test), nor between T0-control and T28-control (absence of thermal stress).

Looking at the factorial plane (Fig. 2A) focused on genes with $VIP > 1$ (threshold considered as significant in the PLS analysis, Fig. 2B and Table 1), it appears that the ellipses corresponding to the groups of mussels maintained at field temperature (T0-control and T28-control) are superimposed ($p=0.23$), indicating that the expression profiles do not vary over time. For individuals of the test tank at T0, the superposition of the ellipses is also present with the control groups (T0-control and T0-test, $p=0.59$), evidencing that the initial physiological state of each group of mussels was the same and that the exposure time alone did not induce any modification in gene expression, while an increase in inter-individual variability is observed. Finally, after 28 days of exposure, a significant difference in gene expression level between the two-groups was evidenced (T28-control and T28-test, $p=0.014$). However, in relation to this variability, pairwise comparisons failed to evidence significant differences between-conditions. In order to identify the potential source of these variations, we hypothesized that an underlying genetic component could partly explain the increase in inter-individual variability in the group of mussels exposed to 20°C. In a previous study, potential correlations between the phosphoglucosyltransferase genotype and the level of mRNA expression have been shown in the hydrothermal mussel *Bathymodiolus azoricus* exposed to different temperature increases (Boutet al., 2009). In this study, we genotyped the *Glu-5'* locus, which was also used to determine the fine genetic structure of the Kerguelen mussels (Gerard et al., 2015) and showed that the three different genotypes (EE, EG and GG) are represented in the four experimental groups (Table S3). In order to detect a potential difference in mRNA expression depending on the genotype of the individuals, a PCA was performed. The factorial plane evidenced no particular structure within the data, with random distribution of genotypes among

305 exposure conditions (Fig. 3). The MANOVA performed on the individuals with the exposure condition x
 306 genotype combination as independent variable was not significant (Wilks Lambda = 0.557, $p=0.74$).
 307 Other tests done to detect a potential effect of conditioning of individuals (tanks and exposure time) are
 308 not significant, showing that for a given condition individuals are impacted in the same way. Finally, the
 309 genotype effect was assessed at T0, in the test tank ($n=3$ for (EE) and $n=6$ for (EG)). In this last case,
 310 5 significant differences were found between expression levels of the two genotypes: ATP synthase
 311 ($p=0.002$), Big defensin ($p=0.008$), HSP90 ($p=0.037$), mitMDH ($p=0.046$) and PGmut ($p=0.043$). Values
 312 were similar for the 46 other genes. As no mortality related to conditioning or temperature increase was
 313 observed over the duration of the experiment, this suggests that the mussels are capable of withstanding
 314 a relatively large temperature change. In their original environment, mussels are subjected to strong
 315 variations related to the tides (emersion, sudden temperature change, etc.) and their distribution on the
 316 intertidal zone is not only conditioned by these factors but also by the structure of the environment (rock
 317 vs. sand, waves, fresh water input, presence or absence of an algae bed). These constraints influence
 318 the genetic structuring of the mussels (detected with the nuclear marker *Glu-5'*) at the scale of the
 319 archipelago but also at the scale of a site (Gerard et al., 2015). Although this study is purely genetic and
 320 does not provide information on the physiology of these mussel populations, we can assume that
 321 individuals carrying different *Glu-5'* genotype have different responses to environmental variations.
 322 Increase of water temperature is known to induce changes at mRNA expression level as a part of cellular
 323 stress response (CSR) (Evans and Hofmann, 2012), including genes related to various metabolism
 324 processes in order to maintain cell functions (Kültz, 2005; Lockwood et al., 2010, 2015; Somero, 2010,
 325 2020). In the present study, the expression of 51 genes involved in different metabolism pathways was
 326 analyzed (Table S1) and among them, 17 showed significant variations in expression between groups
 327 of mussels exposed to 7.5°C or 20°C at T28 (Table 1). Interestingly, the expression of most of them
 328 decreased after exposure to 20°C and only 4 had higher expression in the same individuals. More
 329 generally, when all the genes are observed (including those with no significant differences in
 330 expression), mRNA expression decreases after exposure to 20°C. Even if the differences are not
 331 significant on the global analysis, it can nevertheless be noted that the lack of significance at this level
 332 is partially explained by the inter-individual variability observed previously (Fig. 2). The decrease in gene
 333 expression and more generally in metabolism in response to stress (temperature, hypoxia, etc.) has
 334 been particularly well described in marine organisms (Storey and Storey, 2004; Anestis et al., 2007,

2008; Boutet et al., 2009) and is presented as an essential mechanism during hibernation, estivation, oxygen deprivation or periods of intense heat stress (see Storey and Storey, 2004 for review). In the case of Kerguelen mussels, exposure to 20°C, although considered a moderate stress, seems to affect the metabolism at the transcriptional level to limit energy cost. At the global level, genes coding for proteins involved in energy metabolism (aerobic and anaerobic) seem to be strongly impacted: 6 have a significant decrease in expression (*glyc phos*, *aconitase*, *TPI*, *octDH*, *enolase* and *aldolase*) and 1 an increase (*ATP synth*) (Table 1), and the other 19 genes involved in energy metabolism show a decrease in expression although not significant. Our results with ATP synthase subunit α are consistent with the fact that response to stress impacts organism metabolism according to its duration and/or intensity and that mussels have to cope with energy demand through ATP production to maintain organism and cell functions (Sokolova, 2013). ATP synthase is particularly involved in this mechanism and is actively regulated at both transcriptomic and proteomic levels. Moreover, the anaerobic metabolic pathways commonly involved in the response to thermal and hypoxic stress in coastal organisms (opine pathways for example, Harcet et al., 2013; Han et al., 2017), are here regulated (significantly or not) in the sense of a decrease in mRNA expression. Indeed, while *LDH* and *cytMDH* expression did not vary significantly, *OctDH* mRNA is significantly less expressed in mussels exposed to 20°C. It can be assumed that these enzymes are regulated at post-transcriptional level to respond to environmental constraints, as is the case here for *cytMDH* which shows an increase in the amount of protein (see section 3.2).

In response to an increase in temperature, the expression of some other selected varies significantly: *Hsp60* (stress response), *mt20* (detoxification) and *HFI* (O_2 sensing) are overexpressed in individuals exposed to 20°C. Several studies have shown a regulation of *Hsp60* mRNA expression in marine bivalves in response to increased temperature, without fully understanding its direct involvement in this response (Snyder et al., 2001; Ding et al., 2018). The other members of the HSP family studied here show either a significant decrease in expression (HSP24 and HSP90) or an absence of variation (HSC71, HSP22 and HSP78). In this work, it would appear that stress proteins are also regulated at the post-transcriptional level.

Interestingly, the MT10 and MT20 metallothionein system is antagonistically regulated, with *mt10* showing a decrease in expression while *mt20* increases in the gills of mussels exposed to 20°C. Although MTs are mainly involved in protection against metals or oxidative conditions (Le et al., 2016), an increase in the level of *mt20* expression has already been shown in thermally challenged mussels

(Hamer et al., 2008; Gourou et al., 2010; Feidantsis et al., 2020). On the other hand, *mt10* has so far rather been considered as having no expression variations (constitutive form as opposed to the inducible form MT20, Dondero et al., 2005; Raspor et al., 2006; Gourou et al., 2010). However, several studies have reported variations in the expression of *mt10* (often an increase) in response to different stresses (Banni et al., 2014; Lacroix et al., 2014; Rossi et al., 2016). It thus appears that Kerguelen mussels have a different response to *mt10* gene expression than other mussels (especially from the northern hemisphere) characterized by a decrease of expression.

Under natural conditions, an increase in temperature reduces the amount of available oxygen, thereby inducing the risk of hypoxia-driven stress response for organisms, which have higher metabolic demand (Vaquer-Sunyer et al., 2008; Breitburg et al., 2018; Roman et al., 2019). During the experiment, the mussels were in constant immersion and the water was continuously aerated; no hypoxic events were thus caused by the temperature increase in the tanks. The regulation of genes involved in oxygen sensing is therefore not linked to a decrease in available oxygen but to the temperature increase itself. In this work, the expression profile of the 5 genes coding for proteins involved in the response to oxidative stress and O₂ sensing is variable: indeed, only Hypoxia-inducible factor 1 α inhibitor (*HFI*) shows a significant increase in its expression, while *CuZnSOD*, *perox 6* and *P-hydrox* show a non-significant decrease and catalase increases non-significantly. These genes have been used as biomarkers in other studies and show significant variations in their level of expression (Banni et al., 2014). The activation pathways by HIF-1 α , although acting promptly during a hypoxic event (Hochachka and Lutz, 2001; Kawabe et al., 2012; Cai et al., 2014; Giannetto et al., 2015), seem to be regulated here by an increase in temperature. However, it is difficult to determine the exact role of HFI in the regulation of the gene cascades involved in the response of mussels. Indeed, HIF-1 α is involved in the regulation of many metabolic pathways under stress conditions, notably glycolysis (Hochachka and Lutz, 2001) and its regulation by an inhibitor at the transcriptional level is complex to relate to the overall response of the organism.

3.2. Proteomic analysis pinpoints a marked recruitment of stress proteins.

In ecotoxicology, 2DE has been widely used to study *Mytilus* species responses to various stresses including temperature, mainly in *M. galloprovincialis*, *M. californianus* or *M. trossulus* (Tomanek and Zuzow, 2010; Ji et al., 2013; Gomes et al., 2014; Xu et al., 2016) but also in *M. edulis* (Apraiz et al.,

2006; Sheehan and McDonagh, 2008; Letendre et al., 2011; Hu et al., 2014; Péden et al., 2016; Péden et al., 2018). We thus conducted a 2DE proteomic analysis of gills collected from mussels maintained at 7.5°C and 20°C.

Eight gels displaying highly reproducible patterns were kept for further analyses (4 per condition). The analysis of the 2DE gel images using Delta 2D (Decodon) produced a final dataset of 957 proteoforms. Analyses revealed that 49 of them were significantly differentially abundant with a 0.01 p-value, of which 37 exhibited higher abundance and 12 were less abundant compared to control (Table 2 and Fig. S1). Most of the proteoforms were up-regulated (67%) and 37 out of 49 were identified by mass spectrometry (Table S4). The overall response corresponds to limited modifications of the gill proteome in terms of number of DAPs and fold changes. Those elements suggest there is no cellular homeostasis disruption in the thermal stress group, only adjustments (Brose et al., 2012).

The interaction map of the 37 aforementioned DAPs was generated from the web-based search STRING database (Fig. 4). The resulting network of protein-protein interaction (PPI) exhibited an enrichment PPI p-value lower than $1.10 \cdot 10^{-16}$, indicating significantly more interactions than randomly expected. In particular, Cellular response to heat stress is the first Reactome Pathways of the network with a false discovery rate of $1.03 \cdot 10^{-7}$ and a cluster of 7 proteins (surrounded in bold in Fig. 4 and Table 2), all presented an increase in abundance in gills of T28-test mussels as compared to the control group: HSP90 A1 (spot 1536), HSP90 B1 (spot 1454), HSC 71 (spot 1608), HSPA 5 (spot 1552), HSP70, spot 1599, HSP56 (spot 1756) and 14-3-3 protein epsilon (spot 2194). All of these proteins, with the exception of the 14-3-3 protein epsilon, belong to the highly conserved Heat Shock Protein family (HSP) which participates in folding and allows protein preservation or renaturation after stress-induced alteration. Upregulation of members of this family is commonly observed during adaptive response to various stresses (Buckley et al., 2001; Kültz, 2003; Hartl et al., 2011; Brose et al., 2012; Powers and Balch, 2013; Kültz, 2020; Somero, 2020). 14-3-3 proteins are adapter proteins implicated in the regulation of a large spectrum of signaling pathways, including glucose metabolism, apoptosis, cell cycle progression, autophagy and cell motility. Other proteoforms involved in response to various stresses were also modified: peptidyl-prolyl cis-trans isomerase B (PPIB, spot 2241), heat shock protein 40 (DNAJB11, spot 1917), heat shock protein 75 kDa, mitochondrial (TRAP1, spot 1588) and a peroxiredoxin (PRDX, spot 2349), the latter being the only one with a decreased abundance. Moreover, the neutral α glucosidase AB is a non-HSP family proteoform involved in folding and quality control in endoplasmic

reticulum and was upregulated. Interestingly, we also observed over-expression of a galectin (spot 1656). Galectins belong to the multi-functional lectin family and may act as stress sensors. For instance, galectins are involved in autophagy and apoptosis and contribute to innate immune response in invertebrates (Bai et al., 2017). Taken together, these results indicate that exposure of mussels at 20°C was sufficient to induce a mild but significant stress response.

In addition to variations in stress proteins, we paid particular attention to proteins involved in cytoskeletal rearrangement and cell trafficking (Fig. 4). Indeed, cytoskeleton is often targeted under stress (Magi et al., 2008; Letendre et al., 2011; Jayasundara et al., 2015; Madeira et al., 2020). Our results showed a significant increase in abundance of β -actin (spot 1913) and tubulin (spot 1721). Expression of other cytoskeletal proteins included an actin filament bundling (fascin, spot 1738), a membrane-cytoskeleton linker protein (radixin, spot 1557), and a protein involved in polymerization and depolymerization of actin filaments (66KDa stress protein, spot 1669). These results are consistent with data from previous studies showing that a temperature stress increases ciliary activity, filtration function and respiratory process in gills of *Mytilus edulis* (Tomanek, 2012). Those changes are accompanied by cell network remodeling. Thus, the cellular traffic category is represented by the sorting nexin 6-like (upregulation, spot 1851) which participates in protein recycling and also in retrograde transport of cargo proteins from endosomes to the trans-Golgi network. This response remains moderate but indicates architectural and transport cellular redesign for adaptation to thermal exposure (Fields et al., 2012). Finally, a wide range of cytoskeletal proteins were observed among the identified DAPs, including other proteins involved in assembly and maintenance of epithelial cells such as tropomodulin 3 (spot 1973), gelsolin (spot 1909) and a predicted centrosomal protein of 135 kDa (spot 1437), the latter being the only one to show a decrease in abundance in comparison with the controls. As discussed above, changes in the cytoskeleton have already been identified in studies examining heat stress in mussels, and in addition to adaptations to cell stress, they may reflect cell proliferation requirements.

Facing thermal stress implies an energy cost that requires cellular energy mobilization (Tomanek, 2012; Somero, 2020). Our findings provide several clues to such demands (Table 2 and Fig. 4): firstly, a rise of cytosolic malate dehydrogenase (spot 2078); secondly, an increase in several mitochondrial actors, namely a NADH ubiquinone oxidoreductase 75kDa (core subunit of respiratory chain complex I, spot 1578), a succinate dehydrogenase [ubiquinone] flavoprotein subunit (major catalytic subunit of respiratory chain complex II, spot 1616), a glutamate dehydrogenase (mitochondrial matrix enzyme

involved in glutamine anaplerosis by catalyzing the oxidative deamination of glutamate to alpha-ketoglutarate and ammonia, spot 1735) and prohibitin (spot 2244), mainly involved in mitochondrial biogenesis and activity (Signorile et al., 2019). These results suggest an increase in mitochondrial metabolism, *i.e.* an enhanced energy supply capacity. This point will be further discussed in the following section.

3.3. Omics data comparison is consistent with enhanced energy supply supporting a persistent cellular stress response.

Although qRT-PCR and proteomic analyses were conducted independently, it is attractive to compare them in order to seek consistency and gain a clearer insight into the cellular processes involved in the response to heat stress.

In this study, a point-by-point comparison results in a significant but expected discrepancy. Hence, only 4 actors are common to both data sets (HSP90, HSC71, HSPA5 and cytosolic malate dehydrogenase); none of them exhibited an identical variation (*i.e.* up/down regulation). Two elements can be raised. First, discrepancy between transcriptomic and proteomic responses has been frequently observed and is fairly widely accepted (Romero et al., 2019). This phenomenon is linked to regulation mechanisms that occur between pre-RNA synthesis and mature protein production (post-transcriptional and post-translational regulations). Moreover, degradation can modulate protein turnover. Second, the size of the two sets of data are not similar because they were not obtained using the same strategy, with gene expression from selected targets whereas 2DE proteomics is a non-targeted approach.

As mentioned above, our study presents different results for candidates followed in transcriptomics and proteomics. However, the integration of the two levels of data can lead to interesting hypotheses. Synthetically, we observed a protein response to heat stress at the protein level, certainly related to the need to maintain protein homeostasis under elevated temperature conditions. At the transcriptomic and proteomic levels, energy metabolism appears to be stimulated, probably to ensure the energy cost of sustaining the chronic heat stress response. It is important to note that HSP protein expression, while often induced in acute stress responses, can also be maintained in more mild or low-level chronic types of stress to accomplish its protective functions (Pei et al., 2012; Ortega et al., 2019; Zhan et al., 2019). This observation is reinforced by the increased abundance of the two proteins, TRAP1 (spot 1588) and prohibitin (spot 2244), which both participate in the protection and maintenance of mitochondrial

function. Furthermore, the qPCR results suggest a general decrease in transcription, which is consistent with a lasting presence of chaperones specialized in the response to thermal stress and, more broadly, to cellular stress. The high level of stress chaperones after 28 days of exposure may therefore reflect less protein degradation. Since the protein turnover is the result of a balance between synthesis and degradation, by reducing degradation in parallel with a decrease in transcription, there is conservation of the general level of proteins, and thereby, of protein homeostasis. This hypothesis is consistent with the absence of any proteasomal component in the DAPs identified at the proteomic level. The decreased abundance of two proteins implicated in post-transcriptional regulation process strengthens this hypothesis: namely, RNA-binding protein 8A (spot 2377), component of the spliceosome required for pre-mRNA splicing and the heterogeneous nuclear ribonucleoprotein L (spot 1661), involved in the formation, packaging, processing, and function of mRNAs.

4. Conclusion

Taken together, our results indicate that mussels from the Kerguelen islands have developed an adaptive response to temperature stress with three main characteristics: i) a long-lasting HSP response supported by ii) increase in energy capacities and iii) cytoskeleton modulations underlying cellular scaffolding and trafficking processes. The response remains moderate, which corresponds to mussels in good physiological condition, *i.e.* living in a low impact site, exposed to slight thermal stress.

Blue mussels are sentinel species used all over the world and there would be a particular interest in integrating subarctic mussels in environmental studies: thanks to their low level of contamination, they constitute a well-preserved equivalent to conventionally monitored populations living in degraded environments. Finally, this would also be an opportunity to gain insight into the impact of solely Global Change on intertidal organisms and test the following hypothesis and its consequences: the gradual temperature rise might lead to adaptation where basal levels of proteins implicated in heat stress response are higher, conferring a greater tolerance to stress at the cost of higher energy demand.

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Figure captions

Figure 1: Experimental design.

Control tank water was maintained at 7.5°C. Test tank water temperature ranged from 7.5°C at T0 to 20°C at T28. Mussels were sampled at T0 and T28 for gene expression (Gene exp., n=10) and at T28 for 2DE analysis (Proteom., n=8).

Figure 2: Gene selection based on the VIP (Variable Importance in the Projection) values.

The PLS-DA (Partial Least Square Discriminant Analysis) was performed on the relative gene expressions of 51 selected genes in the 37 exposed individuals (10 for each conditions à 7.5°C, 9 at T0 for 20°C and 8 at T28 for 20°C). **A.** Exposure conditions were plotted on the factorial plane combining the two first axes of the PLS-DA, explaining 60% of the total variance. Ellipses correspond to a 95% confidence interval for individual distribution exposed in each condition. Lines connect each data point (individual location on the factorial plane) to the gravity center of the exposure condition; **B.** Variables were plotted on a correlation circle, giving the correlation of each variable with the axes of the PLS-DA.

Figure 3: Distribution of genotypes among exposure conditions.

Factorial plane on the two first axes of the PCA (Principal Component Analysis) on the individual x gene expression table (37 lines, 51 rows). Individuals are grouped according to the exposure time and temperature. UNK refers to the individual whose genotype is unknown.

Figure 4: Global protein interaction of the gill proteome of *Mytilus edulis platensis* under mild stress.

Protein-protein interactions predicted using STRING database for the differentially abundant proteins (DAP). Nodes are the DAPs described in Table 2. Interactions are shown by the lines which connect each node. Black circled nodes represent protein involved in the "Cellular Response to Heat Stress" reactome pathway.

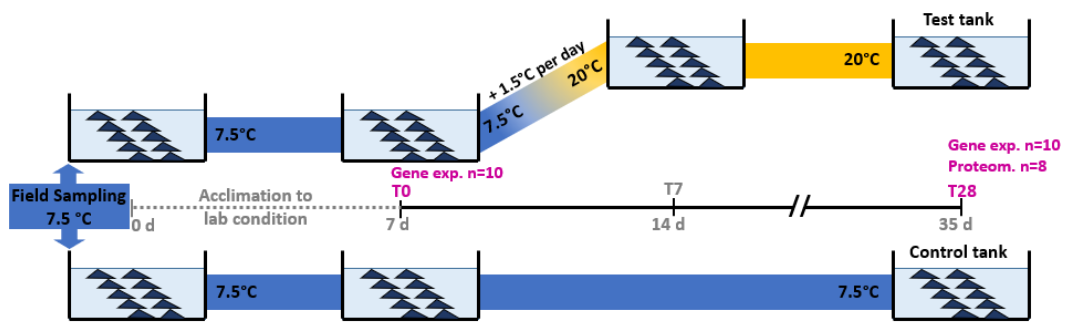


Fig. 1

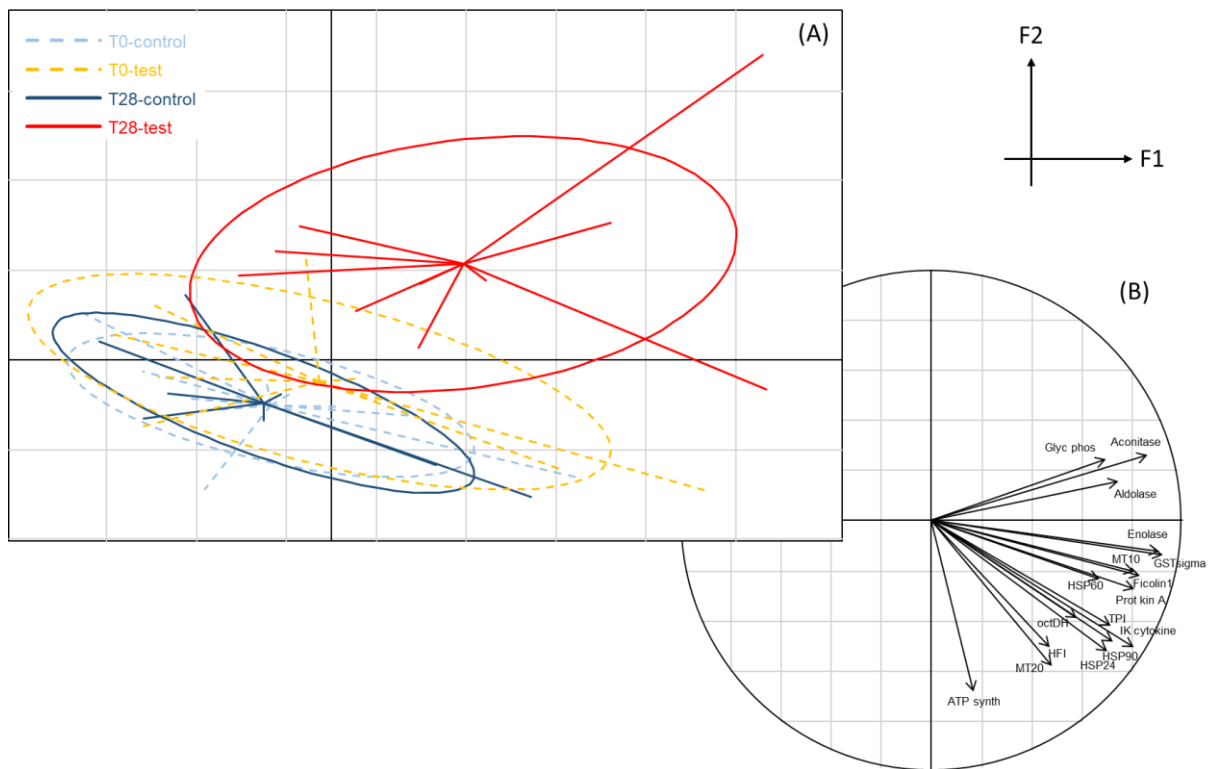


Fig. 2

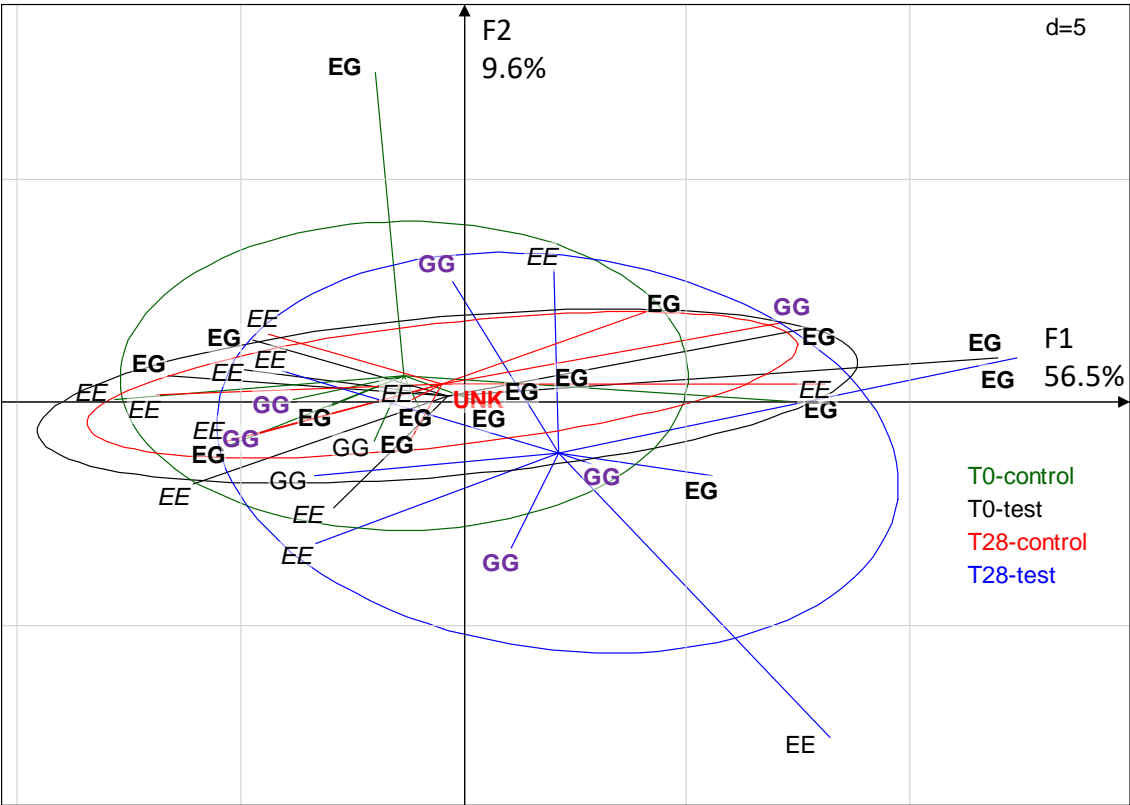


Fig. 3

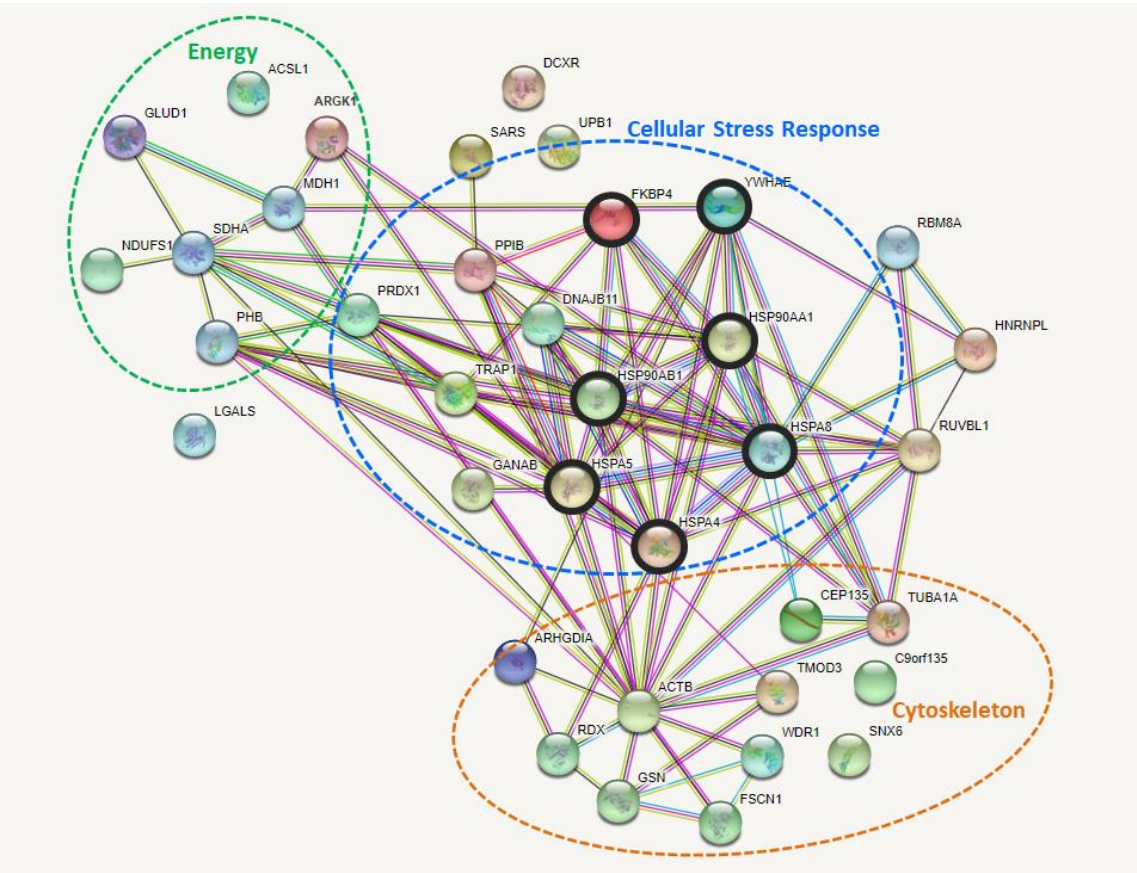


Fig. 4

Table 1. Values of mRNA expression of the different genes in gills of control individuals and exposed to thermal stress (exposed 20°C). VIP values calculated in PLS analysis. Only significant results are presented (VIP>1). Non-significant results are shown in Table S2.

Gene	Control 7.5°C	Exposed 20°C	VIP
<i>MT20</i>	-1,65	2,4	2,22
<i>ATP synth</i>	-1,07	1,45	1,7
<i>Glyc phos</i>	1,45	-3,72	1,45
<i>Aconitase</i>	1,54	-5,62	1,44
<i>IK cytokine</i>	1,26	1,11	1,36
<i>HSP60</i>	-1,75	-1,21	1,33
<i>HFI</i>	1,28	1,52	1,33
<i>TPI</i>	1,72	1,29	1,31
<i>Ficolin 1</i>	1,39	-1,21	1,29
<i>Prot kin A</i>	1,16	-1,14	1,19
<i>HSP24</i>	2,57	1,37	1,19
<i>MT10</i>	1,68	-1,17	1,17
<i>OctDH</i>	1,64	1,1	1,15
<i>HSP90</i>	1,04	-1,15	1,14
<i>Enolase</i>	1,18	-1,31	1,1
<i>Aldolase</i>	1,61	-1,57	1,09
<i>GSTsigma</i>	-1,19	-4,32	1,05

Spot Number	obs Mr (Da)	obs pI	Ratio	Identification	Abbreviation
ID1600	72686	5.78	260%		
ID2378	21109	5.33	234%		
ID1557	82031	5.33	183%	Radixin	RDX
ID1448	119923	5.78	176%	Neutral alpha-glucosidase AB	GANAB
ID1973	38812	4.97	166%	Tropomodulin-3	TMOD3
ID1796	50618	5.78	145%		
ID1699	60397	6.76	136%	Serine-tRNA ligase, cytoplasmic	SARS 1
ID1756	55609	5.32	132%	Heat shock protein 56	FKBB4
ID1632	69964	5.79	124%		
ID1616	71464	5.56	114%	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	SDHA
ID1536	88992	5.00	106%	Heat shock protein 90 A1	HSP90AA1
ID1851	46233	6.05	106%	Sorting nexin 6	SNX6
ID1599	73619	5.58	85%	Heat shock protein 70	HSPA4
ID1656	69872	5.05	83%	Galectin	LGALS
ID1588	76523	5.34	82%	Heat shock protein 75 kDa, mitochondrial	TRAP1
ID1669	63860	6.77	80%	Actin-interacting protein 1,66 kDa stress protein	WDR1
ID1578	77961	5.32	75%	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	NDUFS1
ID1587	74975	6.05	70%	Long-chain-fatty-acid--CoA ligase 1	ACSL1
ID2045	34710	8.00	69%		
ID1735	55712	7.10	61%	Glutamate dehydrogenase, mitochondrial	GLUD1
ID1785	49985	6.05	53%	RuvB-like 1	RUVBL1
ID1909	42203	4.83	52%	Gelsolin	GSN
ID1454	110405	4.94	42%	Heat shock protein 90 B1	HSP90AB1
ID1917	41315	5.60	41%	Heat shock protein 40	DNAJB11
ID1552	78605	4.98	41%	Heat shock protein 70 protein 5	HSPA5
ID2244	25927	5.33	41%	Prohibitin	PBH
ID1738	55602	5.85	40%	Fascin	FSCN1
ID1608	69940	5.28	39%	Heat shock cognate 71	HSPA8
ID2287	23857	5.15	35%	Rho GDP-dissociation inhibitor 1	ARHGDIA
ID2001	36624	6.00	32%		
ID2078	33100	6.00	31%	Malate dehydrogenase, cytoplasmic	mdh1
ID1721	53457	5.18	29%	Tubulin alpha-1 A chain	TUBA1A
ID1913	39362	5.32	25%	Actin beta chain	ACTB
ID2194	27756	4.56	21%	14-3-3 protein epsilon	YWHAE
ID1911	41744	6.30	-30%	Beta-ureidopropionase	UPB1
ID2009	36800	5.60	-36%		
ID2016	35479	8.00	-37%	Arginine kinase-like protein-1	ARGK1
ID2054	34759	5.60	-43%		
ID2377	20994	4.99	-46%	RNA-binding protein 8A	RBM8A
ID2030	35626	5.60	-48%		
ID2238	25992	7.50	-52%	L-Xylulose reductase	DCXR
ID2230	26288	6.15	-52%	C9orf135 protein	C9orf135
ID2349	22468	7.63	-53%	Peroxiredoxin	PRDX
ID2241	26073	7.00	-54%	Peptidyl-prolyl cis-trans isomerase B	PPIB
ID2393	21064	8.00	-60%	Unnamed protein product	
ID2360	21674	5.27	-64%		
ID2222	26845	5.70	-66%		
ID1437	136826	5.56	-68%	Centrosomal protein of 135 kDa, predicted	CEP135
ID1661	67272	7.41	-69%	Heterogeneous nuclear ribonucleoprotein L	HNRNPL

Table 2. Differentially abundant proteins (p<0.01) after thermal stress exposure

Spot numbers as assigned in Figure S1. Ratio correspond to variation in % (T28-test/T28-control). Identifications were performed by Xtandem analyses on spectra obtained by tandem mass spectrometry (For details see Table S3). Mr: molecular mass; pI: isoelectric point; obs: observed value on gel.