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

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16

17

18 **Abstract**

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

31

32 **Key words**

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

34 biomonitoring

35

36 1. Introduction

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

49 Scientific consensus has been reached on Global Change and several scenarios predict significant
50 average temperature increase toward 2100 (Intergovernmental Panel on Climate Change-IPCC,
51 Pachauri et al., 2014; Pörtner et al., 2019). They anticipate not only a global warming, but also an
52 increase of extreme events such as heat waves. One challenge we face is to decipher temperature
53 impact on organisms when combined with pre-existing stress. Marine invertebrates have been widely
54 used as bioindicators of coastal water quality, mainly for monitoring chemical pollution, in order to
55 measure site contamination levels but also to assess biological effects on invertebrate communities
56 (Auffret, 2005; Beyer et al., 2017). The intertidal belt of the Kerguelen archipelago is inhabited by
57 numerous mussel beds. Two species share the same rocky shore habitat: the Antarctic sub-species of
58 blue mussel, *Mytilus edulis platensis* (*M. edulis platensis*) and the ribbed mussel *Aulacomya atra*. Blue
59 mussels are keystone species in coastal ecosystems distributed in many oceans as a result of genetic
60 differentiation within smooth-shelled mussels, now grouped in the so-called *Mytilus* complex. In the
61 Northern Hemisphere, the *M. edulis* complex of species is composed of three genetically well delineated
62 taxa: *M. edulis*, *M. galloprovincialis* and *M. trossulus* (Gerard et al, 2008). The occurrence of blue
63 mussels has also been reported in the Southern Hemisphere, as *M. galloprovincialis* and so-called
64 "native Chilean smooth-shelled *Mytilus*" at the southern tip of the South American continent (Borsa et
65 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

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

99

100 **2. Material and Methods**

101 **2.1. Site description and mussel collection**

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

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

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

119 Once at the Port-aux-Français laboratory (approximately 6h post-harvest), the mussels were divided in
120 homogenous groups of 50 individuals per 50L aerated aquarium tanks containing filtered recirculating
121 seawater maintained on a 12h:12h light cycle (Fig. 1). A 7-days acclimation period was conducted at
122 7.5°C, which corresponds to the water temperature observed in the field. A first sampling was then
123 performed for gene expression analysis (T0-control and T0-test). After acclimation, one group of
124 mussels was maintained at the field temperature while the other group was exposed to a mild thermal
125 challenge. Specifically, water temperature was increased from 7.5 to 20°C over a period of 7 days

126 (+1.8°C per day). Finally, mussels were kept under their thermal conditions with daily follow up of
127 temperature, salinity and pH until their sacrifice at T28 (group T28-control and group T28-test). For all
128 sampling, gills were dissected out, immediately frozen in liquid nitrogen and stored at -80°C until
129 analysis. Our experimental design is based on the individual as the sampling unit (and thus, as the
130 replication level), and the level at which we want to assess variations in gene expression and proteomics.

131

132 **2.2. Gene expression**

133 *2.2.1. RNA extraction and qRT-PCR analysis*

134 Total RNA was extracted from gill tissue using TRI Reagent® solution (Ambion) according to the
135 manufacturer's instructions (n=10 per condition). Three µg of total RNA were reverse transcribed using
136 M-MLV reverse transcriptase (Promega, Madison, WI), random hexamers (Promega) and an anchor-
137 oligo(dT) primer (5'-CGCTCTAGAACTAGTGGATCT₍₁₇₎-3'). The relative mRNA expression of 51 genes
138 selected for their involvement in different metabolisms was estimated by real-time PCR amplification
139 using specific primers (Table S1). A volume of 2.1 µl of each diluted reverse transcription product (1:100)
140 was subjected to real-time PCR in a final volume of 5 µl containing 0.4µM of primers and 1X LightCycler®
141 480 SYBR green I master (Roche, Germany). The amplification was carried out as follows: initial enzyme
142 activation at 95°C for 6 min, then 40 cycles of 95°C for 10 sec, hybridization at 60°C for 30 sec and
143 elongation at 72°C for 30 sec. Calibration curves were obtained using serial dilution of a cDNA pool from
144 12 individuals. In this study, we used a fragment of 28S as a reference gene, because other commonly
145 used reference genes (such as EF1α or actin) are often found regulated in stressed bivalves (Boutet et
146 al., 2004; Tanguy et al., 2004; David et al., 2005). Relative expression of each gene was calculated
147 according to comparative Ct method using the formula: $RQ = 2^{-\Delta Ct}$.

148

149 *2.2.2. Statistical analysis*

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

156 Important genes were selected based on the VIP (Variable Importance in the Projection) values, with a
157 threshold value of 1. Exposure conditions were then plotted on a factorial plane combining the two first
158 axes of the PLS-DA, while variables were plotted on a correlation circle, giving the correlation of each
159 variable with the axes of the PLS-DA. The significance of the exposure condition discrimination was
160 assessed by a permutation test based on a cross-validation test (Westerhuis et al., 2010; Szymańska
161 et al., 2012, RVAideMemoire package version 0.9-75). Here, the structure described by our classification
162 variable (exposure condition) was analyzed using a “null model”. In this model, gene expression data
163 are randomized among the two groups, to generate a structure where chance is the only driver of the
164 dataset. At each iteration, the observed and the random structure are compared, and the model is
165 significant if the discrimination is better with the observed rather than with the random dataset. In a first
166 step, the test was performed to define whether the built model was significant, hence that at least one
167 group is significantly discriminated from the others. Once this first assessment done, a second test
168 performs paired comparisons among the four experimental groups with an FDR (false detection rate)
169 adjustment of p-values, to further explore gene expression differences between each group. All analyses
170 were performed with RStudio version 1.1.463, with R version 3.6.3 (R Core Team 2000, RStudio Team
171 2016).

172

173 2.2.3. *Glu-5'* genotyping

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

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

199

200 **2.3. Proteome analysis and statistical treatment**

201 Protein extraction and two-dimensional analysis were performed as previously described in Péden et
202 al., 2016. Gills of mussels sacrificed at day 28 (two conditions: group T28-control and group T28-test,
203 n=8) were ground using Precellys® Homogenizers and sonicated in a 1:4 ratio of lysis buffer (9 M urea,
204 2% CHAPS, 65 mM dithioerythritol, 0.02% pharmalyte 3-10NL, GE Healthcare and 16 $\mu\text{g}\cdot\text{mL}^{-1}$
205 aprotinin). Cellular debris was removed by centrifugation at 10000x g for 20 min at 20°C and protein
206 concentrations of supernatants were determined according to the method of Bradford with bovine serum
207 albumin as a standard. For 2D electrophoresis, 750 μg of proteins per sample were loaded on non-linear
208 wide-range immobilized pH gradients (IPG strip pH 3-10, NL/18 cm, GE Healthcare). The IEF
209 (isoelectrofocusing) was carried out with a horizontal electrophoresis apparatus (Multiphor, Amersham
210 Pharmacia Biotech). The electric parameters were as follow: 500 V for 0.01 h (gradient), 500 V for 5 h,
211 3500 V for 5 h (gradient) and 3500 V for 9.5 h. After IEF, strips were incubated for 15 min in an
212 equilibration buffer (0.5 M Tris buffer pH 6.8 with 6 M urea, 1% SDS, 26% glycerol) containing first, 30
213 mM dithiothreitol and second, 245 mM iodoacetamide. SDS-PAGE electrophoresis was performed with
214 a Protean plus Dodeca-Cell (Bio-Rad) allowing a parallel run of 12 large gels (12% polyacrylamide, 20

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

227

228 **2.4. MS/MS and protein identification**

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

245 enzyme specificity, trypsin; one missed cleavage permitted; variable modifications, methionine
246 oxidation, cysteine carbamidomethylation; peptide mass tolerance, 10 ppm; fragment mass tolerance,
247 0.5 Da. As filter identification results, the following parameters were used: peptide Evalue, 0.05;
248 minimum number of peptides per protein, 2; protein Evalue -4.0. Protein blast search was done against
249 the non-redundant protein database from NCBI (<https://blast.ncbi.nlm.nih.gov>).

250

251 **2.6. Protein network analysis**

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

262

263 **3. Results & discussion**

264 The average temperature at Kerguelen depends on numerous factors, including depth, localization,
265 seasons, currents, etc. In summer, the typical temperature at a 5m depth is approximately 7-8°C (Féral
266 et al., 2016). In summer, during low tide, however, the temperature in mussel beds can reach more than
267 10-15°C, and possibly more in sunny conditions. The 20°C exposure condition is thus not an unrealistic
268 scenario and is most likely to become a common event given the increase of seawater temperature at
269 Kerguelen. The 28-day period is an experimental condition that more or less represents a semi-chronic
270 exposure often used to study the effect of specific factors on the health status of mussels (Beaudry et
271 al., 2016; Coppola et al., 2018; Lenz et al., 2018, etc.).

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

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

277

278 **3.1. Transcriptional analysis reveals metabolic adaptations**

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

285 Looking at the factorial plane (Fig. 2A) focused on genes with $VIP > 1$ (threshold considered as
286 significant in the PLS analysis, Fig. 2B and Table 1), it appears that the ellipses corresponding to the
287 groups of mussels maintained at field temperature (T0-control and T28-control) are superimposed
288 ($p=0.23$), indicating that the expression profiles do not vary over time. For individuals of the test tank at
289 T0, the superposition of the ellipses is also present with the control groups (T0-control and T0-test,
290 $p=0.59$), evidencing that the initial physiological state of each group of mussels was the same and that
291 the exposure time alone did not induce any modification in gene expression, while an increase in inter-
292 individual variability is observed. Finally, after 28 days of exposure, a significant difference in gene
293 expression level between the two-groups was evidenced (T28-control and T28-test, $p=0.014$). However,
294 in relation to this variability, pairwise comparisons failed to evidence significant differences between-
295 conditions. In order to identify the potential source of these variations, we hypothesized that an
296 underlying genetic component could partly explain the increase in inter-individual variability in the group
297 of mussels exposed to 20°C. In a previous study, potential correlations between the
298 phosphoglucosyltransferase genotype and the level of mRNA expression have been shown in the hydrothermal
299 mussel *Bathymodiulus azoricus* exposed to different temperature increases (Boutet al., 2009). In this
300 study, we genotyped the *Glu-5'* locus, which was also used to determine the fine genetic structure of
301 the Kerguelen mussels (Gerard et al., 2015) and showed that the three different genotypes (EE, EG and
302 GG) are represented in the four experimental groups (Table S3). In order to detect a potential difference
303 in mRNA expression depending on the genotype of the individuals, a PCA was performed. The factorial
304 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

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

372 Under natural conditions, an increase in temperature reduces the amount of available oxygen, thereby
373 inducing the risk of hypoxia-driven stress response for organisms, which have higher metabolic demand
374 (Vaquer-Sunyer et al., 2008; Breitburg et al., 2018; Roman et al., 2019). During the experiment, the
375 mussels were in constant immersion and the water was continuously aerated; no hypoxic events were
376 thus caused by the temperature increase in the tanks. The regulation of genes involved in oxygen
377 sensing is therefore not linked to a decrease in available oxygen but to the temperature increase itself.
378 In this work, the expression profile of the 5 genes coding for proteins involved in the response to
379 oxidative stress and O₂ sensing is variable: indeed, only Hypoxia-inducible factor 1 α inhibitor (*HFI*)
380 shows a significant increase in its expression, while *CuZnSOD*, *perox 6* and *P-hydrox* show a non-
381 significant decrease and catalase increases non-significantly. These genes have been used as
382 biomarkers in other studies and show significant variations in their level of expression (Banni et al.,
383 2014). The activation pathways by HIF-1 α , although acting promptly during a hypoxic event (Hochachka
384 and Lutz, 2001; Kawabe et al., 2012; Cai et al., 2014; Giannetto et al., 2015), seem to be regulated here
385 by an increase in temperature. However, it is difficult to determine the exact role of HFI in the regulation
386 of the gene cascades involved in the response of mussels. Indeed, HIF-1 α is involved in the regulation
387 of many metabolic pathways under stress conditions, notably glycolysis (Hochachka and Lutz, 2001)
388 and its regulation by an inhibitor at the transcriptional level is complex to relate to the overall response
389 of the organism.

390

391 **3.2. Proteomic analysis pinpoints a marked recruitment of stress proteins.**

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

395 2006; Sheehan and McDonagh, 2008; Letendre et al., 2011; Hu et al., 2014; Péden et al., 2016; Péden
396 et al., 2018). We thus conducted a 2DE proteomic analysis of gills collected from mussels maintained
397 at 7.5°C and 20°C.

398 Eight gels displaying highly reproducible patterns were kept for further analyses (4 per condition). The
399 analysis of the 2DE gel images using Delta 2D (Decodon) produced a final dataset of 957 proteoforms.
400 Analyses revealed that 49 of them were significantly differentially abundant with a 0.01 p-value, of which
401 37 exhibited higher abundance and 12 were less abundant compared to control (Table 2 and Fig. S1).
402 Most of the proteoforms were up-regulated (67%) and 37 out of 49 were identified by mass spectrometry
403 (Table S4). The overall response corresponds to limited modifications of the gill proteome in terms of
404 number of DAPs and fold changes. Those elements suggest there is no cellular homeostasis disruption
405 in the thermal stress group, only adjustments (Brose et al., 2012).

406 The interaction map of the 37 aforementioned DAPs was generated from the web-based search
407 STRING database (Fig. 4). The resulting network of protein-protein interaction (PPI) exhibited an
408 enrichment PPI p-value lower than $1.10 \cdot 10^{-16}$, indicating significantly more interactions than randomly
409 expected. In particular, Cellular response to heat stress is the first Reactome Pathways of the network
410 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
411 2), all presented an increase in abundance in gills of T28-test mussels as compared to the control group:
412 HSP90 A1 (spot 1536), HSP90 B1 (spot 1454), HSC 71 (spot 1608), HSPA 5 (spot 1552), HSP70, spot
413 1599, HSP56 (spot 1756) and 14-3-3 protein epsilon (spot 2194). All of these proteins, with the exception
414 of the 14-3-3 protein epsilon, belong to the highly conserved Heat Shock Protein family (HSP) which
415 participates in folding and allows protein preservation or renaturation after stress-induced alteration.
416 Upregulation of members of this family is commonly observed during adaptive response to various
417 stresses (Buckley et al., 2001; Kültz, 2003; Hartl et al., 2011; Brose et al., 2012; Powers and Balch,
418 2013; Kültz, 2020; Somero, 2020). 14-3-3 proteins are adapter proteins implicated in the regulation of a
419 large spectrum of signaling pathways, including glucose metabolism, apoptosis, cell cycle progression,
420 autophagy and cell motility. Other proteoforms involved in response to various stresses were also
421 modified: peptidyl-prolyl cis-trans isomerase B (PPIB, spot 2241), heat shock protein 40 (DNAJB11,
422 spot 1917), heat shock protein 75 kDa, mitochondrial (TRAP1, spot 1588) and a peroxiredoxin (PRDX,
423 spot 2349), the latter being the only one with a decreased abundance. Moreover, the neutral
424 α glucosidase AB is a non-HSP family proteoform involved in folding and quality control in endoplasmic

425 reticulum and was upregulated. Interestingly, we also observed over-expression of a galectin (spot
426 1656). Galectins belong to the multi-functional lectin family and may act as stress sensors. For instance,
427 galectins are involved in autophagy and apoptosis and contribute to innate immune response in
428 invertebrates (Bai et al., 2017). Taken together, these results indicate that exposure of mussels at 20°C
429 was sufficient to induce a mild but significant stress response.

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

449 Facing thermal stress implies an energy cost that requires cellular energy mobilization (Tomanek, 2012;
450 Somero, 2020). Our findings provide several clues to such demands (Table 2 and Fig. 4): firstly, a rise
451 of cytosolic malate dehydrogenase (spot 2078); secondly, an increase in several mitochondrial actors,
452 namely a NADH ubiquinone oxidoreductase 75kDa (core subunit of respiratory chain complex I, spot
453 1578), a succinate dehydrogenase [ubiquinone] flavoprotein subunit (major catalytic subunit of
454 respiratory chain complex II, spot 1616), a glutamate dehydrogenase (mitochondrial matrix enzyme

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

460

461 **3.3. Omics data comparison is consistent with enhanced energy supply supporting a persistent** 462 **cellular stress response.**

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

466 In this study, a point-by-point comparison results in a significant but expected discrepancy. Hence, only
467 4 actors are common to both data sets (HSP90, HSC71, HSPA5 and cytosolic malate dehydrogenase);
468 none of them exhibited an identical variation (*i.e.* up/down regulation). Two elements can be raised.
469 First, discrepancy between transcriptomic and proteomic responses has been frequently observed and
470 is fairly widely accepted (Romero et al., 2019). This phenomenon is linked to regulation mechanisms
471 that occur between pre-RNA synthesis and mature protein production (post-transcriptional and post-
472 translational regulations). Moreover, degradation can modulate protein turnover. Second, the size of the
473 two sets of data are not similar because they were not obtained using the same strategy, with gene
474 expression from selected targets whereas 2DE proteomics is a non-targeted approach.

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

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

496

497 **4. Conclusion**

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

503

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

511

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517

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523

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822 **Figure captions**

823 **Figure 1: Experimental design.**

824 Control tank water was maintained at 7.5°C. Test tank water temperature ranged from 7.5°C at T0 to
825 20°C at T28. Mussels were sampled at T0 and T28 for gene expression (Gene exp., n=10) and at T28
826 for 2DE analysis (Proteom., n=8).

827

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

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

836

837 **Figure 3: Distribution of genotypes among exposure conditions.**

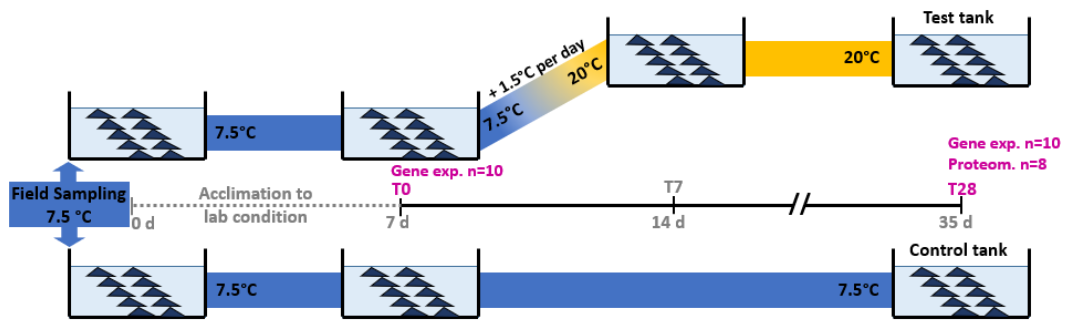
838 Factorial plane on the two first axes of the PCA (Principal Component Analysis) on the individual x gene
839 expression table (37 lines, 51 rows). Individuals are grouped according to the exposure time and
840 temperature. UNK refers to the individual whose genotype is unknown.

841

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

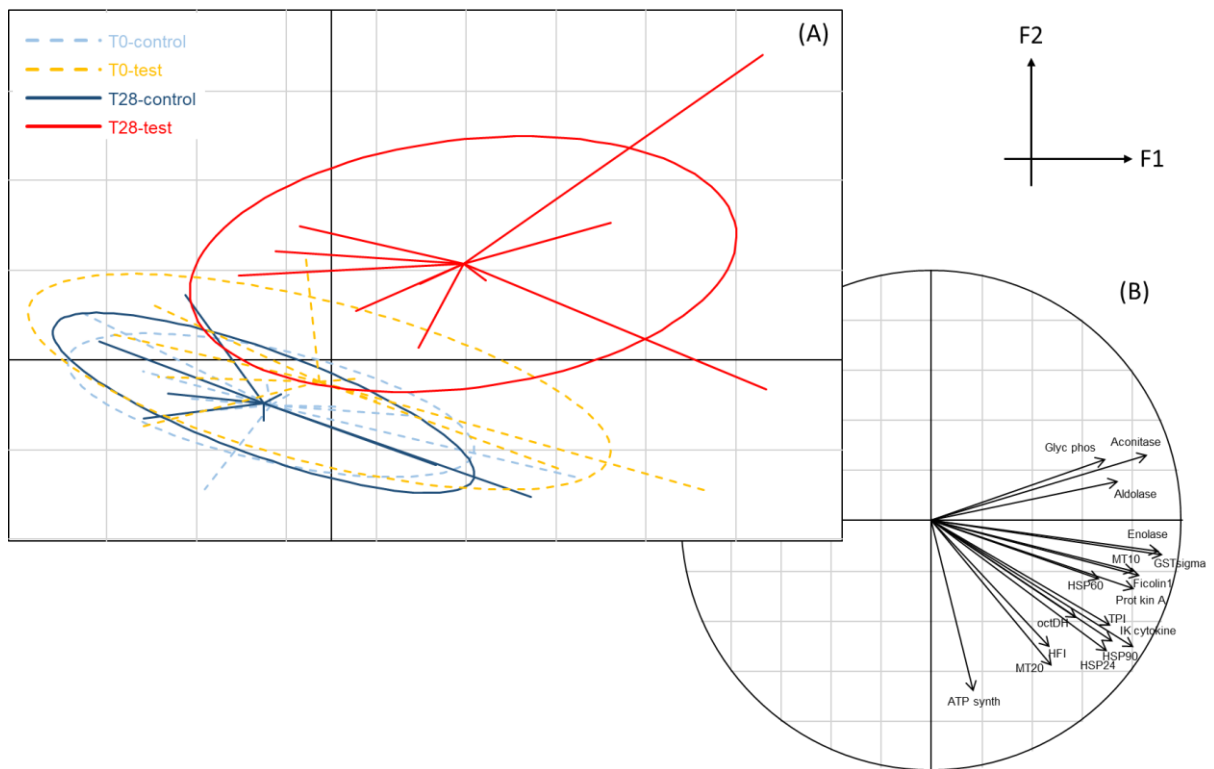
844 Protein-protein interactions predicted using STRING database for the differentially abundant proteins
845 (DAP). Nodes are the DAPs described in Table 2. Interactions are shown by the lines which connect
846 each node. Black circled nodes represent protein involved in the "Cellular Response to Heat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

848



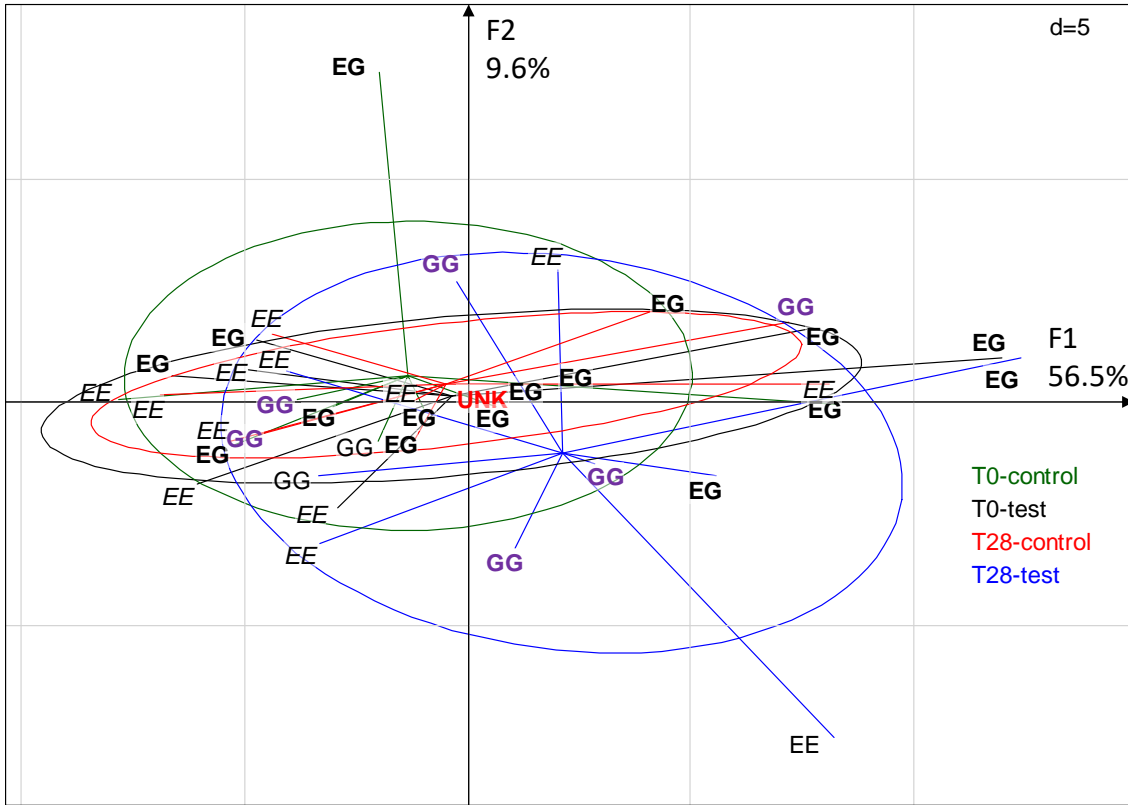
849

850 Fig. 1



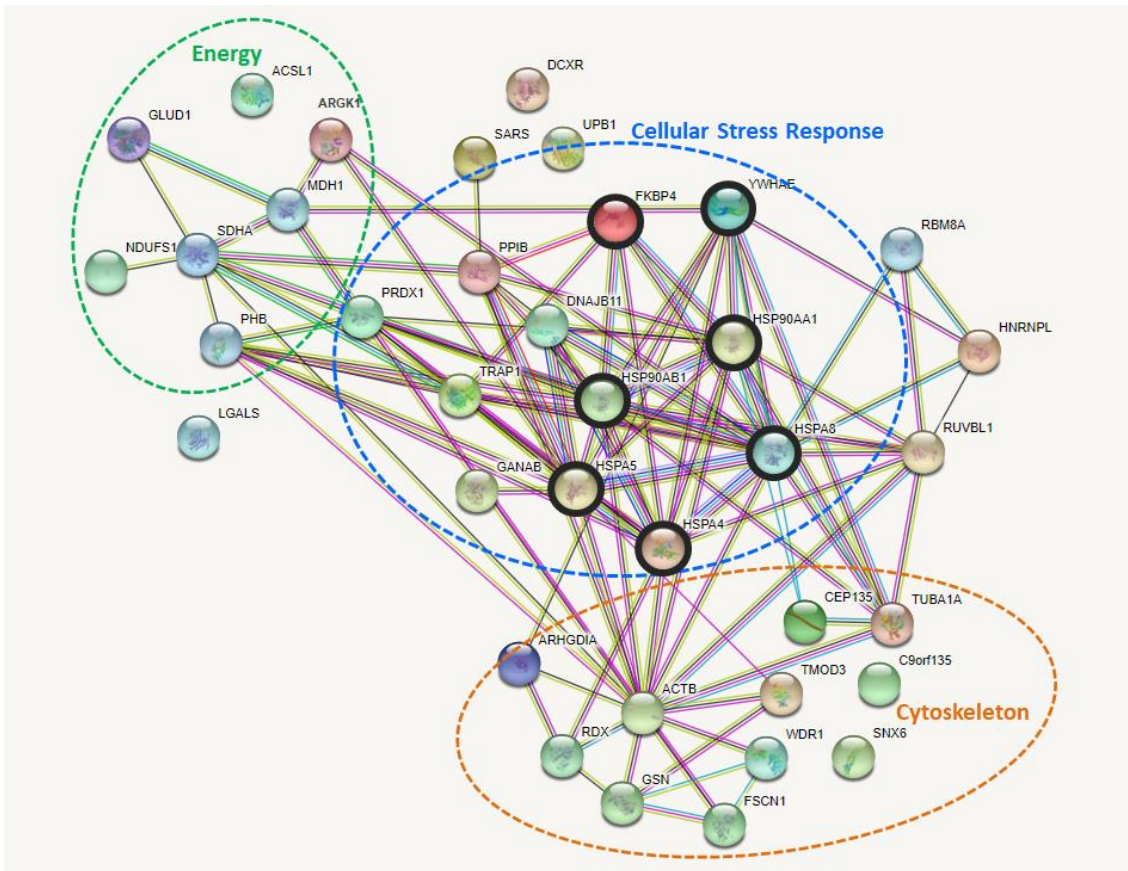
851

852 Fig. 2



853

854 Fig. 3



855

856 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

857

Spot Number	obs Mr (Da)	obs pl	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	ARHGDI1A
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; pl: isoelectric point; obs: observed value on gel.