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1 **Responses of the arcto-boreal krill species *Thysanoessa inermis* to**
2 **variations in water temperature: coupling Hsp70 isoform**
3 **expressions with metabolism**

4

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22

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38

39

40 **Abstract**

41 Recent studies have indicated a metabolic temperature sensitivity in both the arcto-boreal krill
42 species *Thysanoessa inermis* and *T. raschii* that may determine these species' abundance and
43 population persistence at lower latitudes (up to 40°N). *T. inermis* currently dominates the krill
44 community in the Barents Sea and in the high Arctic Kongsfjord. We aimed to increase the
45 knowledge on the upper thermal limit found in the latter species by estimating the CT₅₀ value
46 (19.7°C) (critical temperature at which 50% of animals are reactive) and by linking metabolic
47 rate measurements with molecular approaches. Optical oxygen sensors were used to measure
48 respiration rates in steps of 2°C (from 0°C to 16°C). To follow the temperature-mediated
49 mechanisms of passive response, i.e. as a proxy for molecular stress, molecular chaperone
50 heat shock protein 70 (Hsp70) sequences were extracted from a transcriptome assembly and
51 the gene expression kinetics were monitored during an acute temperature exposure to 6°C or
52 10°C with subsequent recovery at 4°C. Our results showed up-regulation of *hsp70* genes,
53 especially the structurally constitutive and mitochondrial isoforms. These findings confirmed
54 the temperature sensitivity of *T. inermis* and showed that the thermal stress took place before
55 reaching the upper temperature limit estimated by respirometry at 12°C. This study provides a
56 baseline for further investigations into the thermal tolerances of arcto-boreal *Thysanoessa* spp.
57 and comparisons with other krill species under different climatic regimes, especially
58 Antarctica.

59

60 **Introduction**

61 The arcto-boreal krill *Thysanoessa inermis* appears well adapted to the Arctic marine
62 environment that is characterized by low temperatures, strong seasonality in light conditions
63 and hence, primary production (Buchholz et al. 2012; Hop et al. 2006). As a regular expatriate
64 from the Barents Sea, it currently dominates the krill community in West Spitsbergen fjords
65 including the high Arctic Kongsfjord at 79°N (Buchholz et al. 2010; Hop et al. 2006).

66 The Kongsfjord is mainly influenced by two different water masses, which determine the
67 abiotic conditions (e.g. temperature, salinity, nutrients) within this ecosystem: the cold Arctic
68 current and the warm West Spitsbergen Current. The West Spitsbergen Current is the major
69 transporter of heat from the Atlantic to the Arctic, which intrudes into the fjord during the
70 Arctic summer (Hop et al. 2006; Svendsen et al. 2002).

71 During the last decade, continuous hydrographic studies have indicated a climatic shift
72 within the Arctic, i.e. a transition to a warmer state attributed to the increasing influence of
73 warm Atlantic water masses (Polyakov et al. 2007; Spielhagen et al. 2011). This has been
74 particularly documented for the Kongsfjord ecosystem over the last three years. During this
75 time, the inner part of the Kongsfjord remained ice-free over winter (e.g. in 2011/2012,
76 2012/2013 and 2013/2014; personal communication AWIPEV station leader Rudolf
77 Denkmann) documented by mean sea surface temperatures $> 0^{\circ}\text{C}$ from October to January
78 (e.g. for 2012/2013 and 2013/2014 see COSYNA ferrybox at Spitzbergen operated by AWI
79 and HZG Geesthacht; <http://codm.hzg.de/codm>). Furthermore, exceptionally warm water
80 temperatures of up to 8°C were recorded during the summer of 2014 (July/August;
81 ref. COSYNA ferrybox) which were two degrees higher compared to the mean summer
82 temperatures in 2012 and 2013 but almost double when compared to the maximum values of
83 $3 - 4^{\circ}\text{C}$ reported a decade ago (Svendsen et al. 2002).

84 Remarkably, population persistence at lower latitudes, i.e. in particular the abundance of
85 the *Thysanoessa* species, *T. inermis* and *T. raschii*, was found to be determined by ambient
86 water temperatures (Coyle et al. 2011; Hunt et al. 2011). Furthermore, a recent study of
87 Huenerlage and Buchholz (Huenerlage and Buchholz 2015) highlighted the thermal metabolic
88 sensitivity of both *Thysanoessa* species as a potential explanation for the species' restricted
89 biogeographic distribution. At the whole animal level, the authors found that the species were
90 not able to compensate metabolic oxygen demands when ambient temperatures exceeded
91 12°C ; i.e. the species were not able to maintain cardiac activity at temperatures $\geq 12^{\circ}\text{C}$
92 possibly due to a limited capacity for oxygen uptake and/or oxygen transport mechanisms.

93 Nevertheless, there is a clear need for further investigations at the molecular level (i.e.
94 induction of molecular chaperones) to understand the underlying constraints at the cellular
95 level that can provide early indicators of chronic stress and long term survival capacities
96 Accordingly, questions arise about whether the specimens are merely “passively tolerating”
97 increased temperatures or whether the thermal limit of long-term survival may be already
98 reached at temperatures < 12°C.

99 If the species’ long-term thermal limit is below 12°C, then the current summer water
100 temperatures of up to 8°C (see above) may already come close to the true thermal limit of the
101 arcto-boreal *Thysanoessa* species. Hence, in the future, these species may experience
102 maximum water temperatures close to their thermal limit, which may negatively affect their
103 overall metabolic performance and as a consequence, regional persistence.

104 In this context, our first aim was to estimate the upper critical temperature limit of *T.*
105 *inermis* (CT₅₀ = critical temperature at which 50% of animals are reactive) and to confirm the
106 Arrhenius break temperature from temperature dependent respiration curves. These data were
107 supplemented by a molecular approach based on the monitoring of expression of HSP70 gene
108 family members, which are considered as traditional markers of thermal shock. This first
109 study of the heat-induced gene expression of the molecular chaperone heat shock protein 70
110 (Hsp70) may help to understand the thermal adaptive (i.e. survival) capacity of *T. inermis*
111 within the changing ecosystem of the high Arctic Kongsfjord and increase the knowledge on
112 the adaptive potential of this species with regard to its physiological reaction to sudden
113 temperature exposure.

114

115 **Material and Methods**

116 **Sample collection**

117 Krill (*Thysanoessa inermis*) were sampled in late summer 2012 (August 17th – 28th) on-
118 board the Kings Bay AS workboat MS Teisten in the inner part of the high Arctic Kongsfjord
119 (W-Spitsbergen) at 78.95°N, 12.33°E. A 1m² Tucker trawl (1000 µm mesh size and soft cod-
120 end bucket) was deployed at a speed of two knots.

121 Immediately after being caught, adult *T. inermis* were transferred to aerated aquaria
122 containing filtered seawater (0.2 µm) and kept at 4°C in dim light before use in the
123 experiments (respiration measurements and heat shock experiment, see below).

124 No specific permissions were required for these locations and field activities concerning
125 sampling zooplankton, which includes the named Euphausiid species that are not endangered
126 or protected species.

127 **Illumina sequencing**

128 The sequencing process included total RNA extraction from 3 whole animals (one control
129 and two treated (6 and 10°C)) and 60 eyestalks using the SV Total RNA Isolation System
130 (Promega, Madison, WI, USA). Sequencing was conducted by the McGill University and
131 Génome Québec Innovation Centre (Montréal, Québec, Canada) following the manufacturer's
132 instructions (Illumina, San Diego, CA). These data have been submitted to the SRA-EBI with
133 Accession number (SAMN04001594).

134 **RNA-Seq data sets**

135 The cDNA library was sequenced to produce 150bp paired-end reads. Raw reads were
136 filtered with removal of low-quality and low-complexity sequences and trimmed using
137 FASTX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/index.html). The reads were trimmed
138 and filtered using a quality threshold of 25 (base calling) and a minimal size of 60bp. Only
139 reads in which more than 75% of nucleotides had a minimum quality threshold of 20 were
140 retained. Afterwards, rRNA contaminants were removed using ribopicker (Schmieder et al.
141 2012). Adapter ends were cleaned using cutadapt (version 1.01 – (Martin 2011)). Finally the
142 whole quality control process was checked using fastQC (version 0.10.01
143 <http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/>).

144 The resulting assembly was produced using the "de novo" transcriptome assembler
145 Trinity (release 2013-02-25 – (Grabherr et al. 2011)). Finally, reads were remapped on the
146 full transcriptome using Bowtie (version 0.12.8 – (Langmead et al. 2009)) and relative
147 abundances were estimated using RSEM (version 1.2.0 – (Li and Dewey 2011)) to get the
148 FPKM (Fragments per kilobase of exon per million fragments mapped) values and thus
149 identify the low coverage contigs (FPKM<1) and rare isoforms (<1%) that were excluded
150 later from the analysis (both software programs were launched through the Trinity package
151 Wrapper filter_fasta_by_rsem_values.pl).

152 Peptide prediction was performed using Transdecoder (Haas et al. 2013). Sequence
153 similarity searches (blastp of the Transdecoder predicted peptides) were conducted against the
154 UniProt-Swiss-Prot database (release 2013-09). Peptide signal prediction was performed
155 using signalP v4.0 (Petersen et al. 2011). Transmembrane peptide detection was performed
156 using TMHMM v2.0c (Krogh et al. 2001). Protein domain searches was conducted using

157 hmmscan from the hmmer v.3.1b1 suite against the Pfam-A database release 27.0 (Finn et al.
158 2015). Finally functional annotation was produced using the Trinotate pipeline
159 (<http://trinotate.github.io> described in (Haas et al. 2013). GO annotation plotting was carried
160 out using WEGO (Ye et al. 2006) and GO slim analysis mapped against the previously
161 obtained GO annotation against the generic GO slim ontology using custom R and Perl
162 scripts.

163 **Critical temperature estimation**

164 The experimental protocol was identical to that used on the Antarctic species *E.*
165 *crystallorophias* and *E. superba* (Cascella et al. 2015). After acclimation of ~ 24 hours at 4°C
166 in the main aquarium, actively swimming animals were selected for experiments (n=41).

167 The rate of temperature increase in this experiment was 1°C every 10 minutes. The
168 animals were maintained in the experimental tank until they were no longer able to respond to
169 tactile stimuli of a probing rod. At this point, it was considered that the critical temperature
170 limit had been reached and the animals were taken from the aquarium and snap frozen in
171 liquid nitrogen. The CT₅₀ was considered as the temperature at which survival of the
172 experimental animals declined to 50%. This was determined through the non-linear curve
173 fitting option in JMP10 (SAS). The survival curve used was: $Survival=c/(1+(T/CT_{50})^b)$ where
174 c is the plateau value before the sharp decrease, CT₅₀ is the temperature at which 50% of
175 mobile animals is reached, and b is a sigmoidicity coefficient. The program explores the
176 different values of these three parameters, and calculates a Chi-square value. While exploring
177 the different parameter values, the program aims at minimizing Chi-square and converges
178 towards a value for each parameter (standard error provided).

179 **Respiration measurements**

180 Twelve hours after capture, *T. inermis* specimens were randomly chosen for the
181 respiration measurements. In groups of 10 to 20 individuals, the specimens were brought to
182 the final experimental temperatures (0, 2, 6, 8, 10, 12, 14 or 16°C), at a rate of 1°C h⁻¹
183 followed by a 12 h acclimation at the constant experimental temperature. To avoid starvation
184 effects on the metabolic rates, the total exposure times before measurement were not longer
185 than 36 h (e.g. at 16°C).

186 After acclimation, the specimens were individually incubated in closed tubular respiration
187 chambers (Perspex; 20 ml) specially designed for measuring routine rates in krill (Huenerlage
188 and Buchholz 2013; Werner et al. 2012). The chambers were filled with filtered seawater at
189 the experimental temperature and stored in a water bath in a temperature-controlled

190 refrigerator. Oxygen consumption ($\text{mg O}_2 \text{ L}^{-1}$) was monitored every 30 seconds using a 10-
191 channel optode respirometer (Oxy-10 Mini; PreSens Precision Sensing, Germany). This
192 apparatus enabled the measurement of up to 8 individuals in parallel. Two chambers were left
193 blank (without a specimen) and served as controls. After the experiments, the specimens were
194 weighed (mg) and measured for size (from the front of the eyes to the tip of the telson to the
195 nearest mm).

196 **Characterization of Hsp70 isoforms and cDNA cloning**

197 To confirm the Illumina transcriptome assemblies and verify the identified *Hsp70* contigs,
198 nested PCR and sequencing were performed before evaluating the kinetics of *Hsp70*
199 expression in the heat shock experiments.

200 Ribonucleic acid (RNA) was isolated from the abdominal muscle of individual *T. inermis*
201 specimens following the RNeasy® protocol (ref. Qiagen N.V., Netherlands). The
202 concentrations of total RNA were determined photometrically at 260 nm using a Nanodrop®
203 (Thermo Fisher Scientific, USA). RNA purity was checked using the A260/A280 ratio (i.e.
204 absorbance at 260 nm to the absorbance at 280 nm).

205 Purified RNA (1 μg) was retro-transcribed into single stranded complementary
206 deoxyribonucleic acid (cDNA) using SKdT primers (Roche, France) and the M-MLV Reverse
207 Transcriptase kit (Affymetrix USB®, USA) according to manufacturer's instructions. The
208 *Hsp70* isoforms were PCR amplified from the cDNA using specific primers that were
209 designed from sequences obtained from the Illumina assembly. Four pairs of PCR primers
210 were used for each gene in order to clone each isoform in overlapping 1000 base pair sections
211 to facilitate full length sequencing of the whole gene. PCR products were gel purified and
212 amplicons were inserted into the pGEM®-T Vector (Promega Corporation, USA). Plasmids
213 were transformed into DH5 α bacteria (*Escherichia coli*; Life Technologies™, USA).
214 Transformed bacteria were selected and positive clones were verified by PCR. Plasmids were
215 then extracted and sequenced with the same primers as before.

216 **Heat shock experiments**

217 The heat shock experiments were performed 24 h after capture. The experiment was
218 started by immediately transferring ~ 200 *T. inermis* from the lab maintained at 4°C to one
219 aquarium (30 L) containing aerated seawater at 6 or 10°C ('heat shock'). After 3 or 6 hours,
220 one set of animals ($n = 50$) were returned to the control temperature tank at 4°C for recovery.
221 The recovery lasted 6 hours. During the heat shock and recovery time, sub-samples of 10
222 specimens were taken every 1.5 hour or every two hours, respectively. The individuals were

223 snap frozen in liquid nitrogen and stored at -80°C until further analysis at the Station
224 Biologique de Roscoff, France.

225 In parallel to the experiment above, one group of *T. inermis* was kept at 4°C and served as
226 the control, i.e. was sub-sampled synchronously with the specimens from the heat shock
227 experiment.

228

229 **qPCR analysis**

230 The RNA was extracted as described above. Messenger RNA (mRNA) levels of the
231 *Hsp70* isoforms were determined by reverse transcription qPCR amplification. Reactions
232 were performed in a 5 µl total volume containing 2.1 µl of diluted reverse transcription
233 product (1:200), 0.4 µmol of each specific primer and 2.5 µl of SYBR Green I master mix
234 (Roche, France). The amplification was carried out at 95°C for 15 min, then in 55 cycles at
235 95°C for 10 sec and at 60°C for 30 sec. A dissociation curve was generated and PCR
236 efficiency was estimated for each primer pair. All primer pairs tested generated a single peak
237 in the dissociation curve and a PCR efficiency of 80-100%. Data were analyzed with the
238 LightCycler 480 software (Roche, France). The *RPL8* gene was chosen as a reference gene
239 using the BestKeeper algorithm (Pfaffl et al. 2004) after testing *EF1α*, *18S*, *RPL8*, and
240 *GAPDH* as potential normalizing housekeeping gene. *Hsp70* expression was subsequently
241 normalized to this reference. 6 to 10 animals were used for each point.

242 **Data analysis**

243 Metabolic rates were normalized to one mg fresh weight (FW) and expressed in µmol per
244 hour (h⁻¹). The Arrhenius break temperatures (ABT) of the temperature dependent respiration
245 curve was estimated from the Arrhenius plots of the corresponding respiration rates (Dahlhoff
246 et al. 1991). A one-way ANOVA with post-hoc Dunnett test was performed to test for the
247 temperature influence on the specimens' respiration rates.

248 Differences of the mean normalized expression (MNE) of the five *Hsp70* isoform genes
249 (B, C1, C2, D and E) over the time course of the experiment were analyzed using a non-
250 parametric Kruskal–Wallis test. Relative gene expression (fold *Hsp70* expression) was
251 calculated from the MNE of test specimens (heat shock at 6 or 10°C for 3 or 6 hours and
252 subsequent recovery at 4°C) divided by the MNE of control specimens (kept at 4°C control
253 temperature).

254 Statistical analyses were carried out using GraphPad Prism 6 (GraphPad Software, Inc.,
255 USA). The significance level was set at $p < 0.05$.

256 **Phylogenetic reconstruction**

257 Phylogenetic reconstructions were carried out on 68 Hsp70 family proteins including
258 Grp78 and mitochondrial isoforms from different crustacean species, using Bayesian
259 Inference (BI) methods. Bayesian analysis was performed using MrBayes 3.1.2 with four
260 chains of 10^6 generations, trees sampled every 100 generations, and the burning value set to
261 20 % of the sampled trees. Protein sequences were analyzed with a mixed amino-acid model
262 (Ronquist and Huelsenbeck 2003).

263 **Results and Discussion**

264 **CT₅₀**

265 During the temperature challenges, *T. inermis* did not seem to be affected up to a
266 temperature of 18°C. The interpretation of the curve, i.e. the loss of mobility as a function of
267 temperature, shows a CT₅₀ value of 19.7°C ± 0.09°C (Fig. 1). However, this value provides
268 little clue in direct relation to the environmental conditions that the animal would encounter.
269 It is nevertheless helpful to compare pelagic species from different climates for example,
270 different species of krill. This experiment, when conducted in an identical manner on animals
271 from other ecological or climatic backgrounds enables differential sensitivities to be
272 highlighted, which serve as comparative basis and characterize different resilience capabilities
273 (Peck et al. 2009; Terblanche et al. 2011).

274 *T. inermis* therefore appears to be very thermo-tolerant with this high CT₅₀ value. Indeed,
275 this temperature is higher than those observed under the same experimental conditions in
276 Antarctic *Euphausia* species (Cascella et al. 2015). However, in the context of this
277 comparison, it is necessary to relate these values to habitat temperature. The starting
278 temperature of the experiment was 4°C for the boreal species, therefore this species survived
279 up to an additional 15.7°C of warming, a value which is very similar to the CT₅₀ of the
280 Antarctic species whose ambient temperature is around 0°C. Thus both Northern and
281 Southern species appear to have similar resiliencies to temperature.

282 ***T. inermis* transcriptome assembling**

283 A total of 207,011,779 raw sequences with read lengths of 150 bp were generated. After
284 data cleaning to remove adapters and quality control, 205,445,915, high quality reads were
285 obtained. These were used to produce a first assembly of 340,890 transcripts (corresponding
286 to 214,624 Trinity 'genes') from 201 to 19,191 bp with an average length of 904 bp and a
287 median length of 411 bp. The majority (90.7%) of the cleaned reads were successfully

288 mapped back to the full transcriptome indicating strong support for the assembly. Lowly
289 expressed transcripts (FPKM < 1) and rare isoforms (< 1%) were excluded from the initial
290 assembly leading to a filtered assembly of 54,319 transcripts (corresponding to 34,066 Trinity
291 'genes') from 201 to 18,966 bp with an average length of 1222 bp and a median length of 723
292 bp.

293 **Putative functional analysis of the *T. inermis* transcriptome**

294 20,626 proteins were predicted in the transcriptome. 11,124 of them had blastp matches
295 (with an e-value < 10⁻⁴) against the UniProt-Swiss-Prot database (Online Resources 1A).
296 Particular attention was paid to the GO terms "response to stress" (Online Resources 1B). The
297 ontology GO006950 represented 7.2% of GO designations and reached 11% after the GO-
298 Slim annotation. This category included different members of the Hsp family, including the
299 Hsp70s.

300 **Structure of Hsp70 isoforms**

301 To estimate the onset of thermal stress in an organism, it is necessary to analyze the
302 effects of temperature at the molecular scale. The primary effect of high temperature is
303 denaturation of proteins and destabilization of cellular homeostasis, which leads to cell death
304 and finally to the death of the organism. Heat shock proteins (HSP) are well known to
305 counteract these deleterious effects, facilitating the refolding of proteins. There are many
306 HSPs classified according to their weight in Kilo Daltons (from 10 kDa to 110kDa). Within
307 this family, the heat shock protein 70 kDa (Hsp70) are the most studied. They are generally
308 highly transcribed in response to heat shock, where they act as chaperone proteins and
309 orchestrate the recruitment of other HSPs. They are, by their activity, largely responsible for
310 the thermal tolerance of an organism. Indeed, the absence of these molecules can significantly
311 decrease tolerance capacity (Bettencourt et al. 2008).

312 The Hsp70s are traditionally separated into one of two categories; the first contains the
313 inducible Hsp70s called so because their expression is induced during stress. The second
314 comprises the Hsc70s (heat shock cognate), often called constitutive because they are
315 constantly expressed in the cell, at a basal level.

316 Six isoforms of Hsp70 were extracted from transcriptomic data. They were named B, C1,
317 C2, D, E and F according to their sequence similarities and in agreement with orthologous
318 comparisons with the Southern Ocean species (Casella et al. 2015) (Fig. 2). Unlike the
319 Southern Ocean species, the A form was not identified in the transcriptome data, while two
320 isoforms C (C1 and C2) have been characterized in *T. inermis*. The F isoform was

321 subsequently extracted from the transcriptome assembly, but has not been confirmed by RT-
322 PCR and no expression studies have been carried out to date. However, the orthologous
323 sequence has been extracted from a new *E. superba* transcriptome (pers. data, unpublished),
324 validating the identity of this sequence and the identification of six krill Hsp70 paralogues to
325 date.

326 The different isoforms were generally very similar to each other in terms of their primary
327 sequence with high percentage identities ranging from 71 to more than 85% at the amino acid
328 level. The exception was the D form with 42-44% identity, which was putatively designated
329 as the mitochondrial form Hsp74 (Cascella et al. 2015). These isoforms clearly associated
330 with potentially orthologous sequences previously identified in *Euphausia superba*
331 (ThiHsp70B/EusHsp70B = 96%; ThiHsp70C1-C2/EusHsp70C = 77-85%;
332 ThiHsp70D/EusHsp70D = 90%; ThiHsp70E/EusHsp70E = 91%; ThiHsp70F/EusHsp70F =
333 93%). The B, E and F isoforms were structurally related to constitutive isoforms because of
334 the tetrapeptide repeat motif (GGMP), which was present 3, 1 and 4 times respectively. The
335 C1 and C2 isoforms were equivalent to the inducible forms due to the presence of additional
336 tetrapeptide sequences (residues 191-194); however, the C1 form carried a final GGMP,
337 indicating a potential hybrid molecule. All these isoforms had the terminal I/VEEVD as
338 signature motifs indicating to their cytoplasmic localisation.

339 The E and F isoforms were equally represented in the *T. inermis* transcriptome and were
340 structurally designated as constitutive and cytoplasmic. They were present in similar
341 quantities as the mitochondrial D form. This observation overlaps with the FPKM data
342 obtained in *E. crystallorophias* for the D form and partial E form, which was not fully
343 characterized (Cascella et al. 2015). The E form has only been found in one other species with
344 a unique potential orthologous sequence in the crab *Portunus trituberculatus*
345 (ACZ02405.1)(Cui et al. 2010). The latter is annotated as Hsp70 cognate-4 and is also found
346 in various insects where it has been identified from genomic data. The F forms have not yet
347 been identified in any other species.

348 There are two other key differences in the comparison with the Antarctic krill species: the
349 lack of the A form and the presence of two inducible forms. In *Euphausia*, the two A and B
350 forms, which were characterized as structurally constitutive, were the most highly expressed
351 *Hsp70s*. In contrast, in *T. inermis*, only the B form was present. It also is the most highly
352 represented form in the mRNA population of the transcriptome sampled (Table 1). The
353 potential inducible forms in *T. inermis* were not equally represented. The C2 isoform was
354 more represented in the transcriptome than the C1 isoform suggesting different functions.

355 This low level of C1 may also be an explanation for the lack of characterization in *Euphausia*
356 and in other crustaceans. In *T. raschii*, a short sequence potentially related to a C1 form has
357 also been identified (pers. obs. data, unpublished).

358 These qualitatively and quantitatively differential representations of Hsp70 isoforms in
359 boreal and Antarctic krill species may be associated with various response strategies. These
360 could be potentially associated with different environmental stresses or different temperature
361 ranges and stabilities, via the action of selection on gene duplication events and the
362 subsequent sub-functionalization that ensures either their maintenance within the genome or
363 their disappearance (Prince and Pickett 2002).

364 **Molecular phylogeny of the Hsp70 family**

365 To confirm and establish the phylogenetic relationships in crustaceans between the
366 different isoforms of Hsp/Hsc70, 68 sequences of Hsp70 *largo sensu* (Grp78 and
367 mitochondrial isoforms were included) were aligned. A tree was produced using the Bayesian
368 inference method. This tree confirmed the positions of the *Euphausia* isoforms (Cascella et al.
369 2015) and the designations assigned to different isoforms of *T. inermis* previously assigned
370 solely on the basis of their sequence similarities (Fig. 3).

371 The positions of the E and F forms were confirmed and they constitute a sister group to a
372 cluster classically considered as the constitutive isoforms, which includes the Euphausiid A
373 and B forms.

374 The *T. inermis* C isoforms were positioned in a second set, grouping the inducible
375 Hsp70s. The branch lengths of this set attest to a faster rate of evolution of these isoforms
376 compared to the Hsc70s. Although only one C form was characterized in *Euphausia*, two are
377 present in the transcriptome of *T. inermis*. The two sequences are not, as in the case of
378 *Rimicaris exoculata*, present on the same branch of the tree. Indeed, the C1 isoform is
379 positioned at the base of the cluster indicating that it is potentially close to an ancestral form.

380 It is clear from the phylogeny that the group of the inducible forms is numerically less
381 represented than that of the constitutive forms. However, it may simply be that the sampling
382 is not representative. Most of these data arise from transcriptome studies and referring to the
383 FPKM data in the current study the C1 form is very poorly represented compared to the C2.
384 This very low abundance could explain the absence of orthologous forms in the other species.
385 These may well be discovered with more intensive transcriptome sampling or the production
386 of draft genomes in the crustacean in the future.

387 **Metabolic rates**

388 In total, 122 adult *Thysanoessa inermis* were sampled for the respiration measurements.
389 Of these, 46 % were determined as females, 38 % as males and 16 % were determined as
390 neuter due to sexual regression which did not allow for a clear sex determination.
391 The specimens had an average fresh weight of 115.74 ± 4.2 mg and an average size of $24.5 \pm$
392 0.2 mm. There was no significant size difference between the sexes. Furthermore, the
393 respiration rates did not differ between sexes. Therefore the data were pooled for the
394 comparison of experimental temperature effects (Table 2, Fig. 4).

395 The normalized respiratory performance over the experimental temperatures could be
396 divided into two phases (Fig. 4). In the first temperature increment (0 – 12°C), respiration
397 rates increased exponentially. In reference to the 4°C control temperature, the increase was
398 significant from 8°C ($4.7 \pm 0.2 \mu\text{mol O}_2\text{h}^{-1}\text{gFW}^{-1}$ at 4°C vs. $7.8 \pm 0.3 \mu\text{mol O}_2\text{h}^{-1}\text{gFW}^{-1}$ at
399 8°C; Table 1, Fig.1; $p < 0.0001$, $F = 15.2$, one-way ANOVA with Dunnett's Multiple
400 Comparison Test against 4°C control temperature). However, at experimental temperatures
401 beyond 12°C, mean oxygen consumption decreased from $10.2 \pm 0.9 \mu\text{mol O}_2\text{h}^{-1}\text{gFW}^{-1}$ at
402 12°C over $9.5 \pm 0.9 \mu\text{mol O}_2\text{h}^{-1}\text{gFW}^{-1}$ at 14°C to $7.3 \pm 2.1 \mu\text{mol O}_2\text{h}^{-1}\text{gFW}^{-1}$ at 16°C. The
403 tipping point was depicted by the Arrhenius plot and showed the respiratory Arrhenius
404 breakpoint temperature at 12°C indicated by a sharp change in the slope of linear regression
405 (=ABT; Fig. 4).

406 The respiratory response of adult *T. inermis* to experimental temperature change was the same
407 as previously determined (Huenerlage and Buchholz 2015): increasing temperatures resulted
408 in remarkable metabolic disturbance at temperatures exceeding 12°C. This tipping point was
409 determined by the Arrhenius breakpoint temperature (ABT) and consequently, characterized
410 the upper limit of temperature-induced oxygen demand, i.e. the upper pejus temperature limit
411 (TpII) (Frederich and Pörtner 2000) after which metabolism changes from aerobic to
412 anaerobic (Pörtner 2012).

413 However, the respiratory response does not provide a measure for temperature-induced
414 stress at the cellular level. In marine ectotherms, increased ambient water temperature is one
415 of the major factors causing cellular damage due to the denaturation of proteins (Feder and
416 Hofmann 1999; Kültz 2005). Accordingly, species have evolved responses to environmental
417 stressors using molecular chaperones that help to prevent protein degradation and hence,
418 enable them to survive during the periods when the species are exposed to unfavorable abiotic
419 conditions.

420 ***Hsp70* gene expression**

421 In this study, the molecular chaperones ‘heat shock protein 70’ (Hsp70), which are well
422 known as characteristic indicators of cellular stress, were investigated. The expression
423 kinetics of five of the six isoforms were established according to the intensity of the heat
424 shock (6 or 10°C) and the duration (3 or 6h). The responses after a return to the starting
425 temperature (recovery) were also measured every two hours for a total of six hours (Fig. 5).

426 The kinetics showed clearly that the various isoforms did not have identical responses to
427 the heat shocks. Furthermore, the notions of inducibility and constitutivity were not met as
428 designated by sequence similarity and identification of signature motifs. Indeed, the forms
429 assigned as constitutive (B, D, E) showed the most significant fold changes in expression
430 whilst the designated inducible forms (C) appeared as the least expressed, and even repressed
431 (Fig. 5, Online Resource 2).

432 The B isoform did not show any significant increase before three hours of heat shock,
433 regardless of its amplitude, 6°C or 10°C. The kinetics of expression of this isoform showed
434 peaks that suggested regulation by a feedback control as classically expected for an inducible
435 Hsp70. The height of the expression peaks after 3 hours shock was greater at 10°C than at
436 6°C suggesting a relationship between shock intensity and response amplitude. The
437 expression could be further modulated as shown by the different successive peaks. In contrast,
438 the amplitude of the response during the post shock i.e. after a return to 4°C was higher after a
439 shock to 6°C rather than 10°C. The answer after 3 hours of shock might be insufficient to
440 counter the effects of stress and might explain this important secondary response. It is also
441 interesting to note that the maximum fold increases in expression levels were similar whether
442 during the shock and post-shock. Furthermore, the delay of the response could be explained
443 by a constitutively large concentration of Hsp proteins present in the tissues.

444 The response kinetics of the mitochondrial D form also depended on the intensity and
445 length of the thermal shock with a significant up-regulation after 3 hours at 10°C and after 6h
446 at 6°C. Whatever the shock time, elevated expression was most important during recovery,
447 indicating a metabolic disturbance in the mitochondria. Little is known about the behavior of
448 this type of HSP, but this observation would demonstrate that the heat shock disrupted the
449 functioning of the mitochondria requiring extensive repair when returning to normal. Indeed,
450 high temperature increases the metabolism and makes the mitochondria less efficient. More
451 free radicals are produced. If Hsp70 cannot directly repair free radical damage, they might
452 provide stability to mitochondrial and cellular antioxidant enzymes, which may be less
453 efficient at high temperatures as well. The early response to 10°C is consistent with the results

454 obtained from the respirometry experiments highlighting an increased metabolism via the
455 proxy of oxygen consumption and a much greater involvement of the mitochondrial
456 respiratory chain in response to the acute stress.

457 Paradoxically for the isoforms considered as inducible (C1 and C2), the trend of the
458 response to thermal shock appeared stable, or even negative. This decrease could be attributed
459 to a massive recruitment of mRNAs, originally present before the heat shock, for protein
460 synthesis. Much of the response would then consist in replacing the mRNAs involved in
461 translation. On the other hand, the absence of elevated expression may be related to the
462 existence of a base rate quantitatively close to the maximum limit of production of mRNAs
463 encoding these isoforms. Indeed, the quantities measured by qPCR showed, for the C2 form
464 especially, values close to those observed for the B isoform in the controls.

465 The HSP response characterizes a state of stress at the cellular level in an organism
466 (Colson-Proch et al. 2010). However, the response is not the same depending on the species
467 and the stress applied. A few organisms like the Antarctic Notothenioid fish have lost the
468 inducible heat shock response (Hofmann et al. 2000). Whilst others, such as the starfish
469 *Odonaster validus*, or the amphipod *Paraceradocus gibber* (Clark et al. 2008) may have also
470 lost these mechanisms during evolution, as an effective response of HSP70s to warming has
471 yet to be shown in these species. The lack of a typical HSP response for this type of organism
472 cannot be considered as an absence of stress, but rather as an inability to overcome damage
473 caused by a factor, which denatures proteins, such as temperature. This absence is evidenced
474 by a special sensitivity to an increase in their habitat temperature. However, many of these
475 studies evaluated a restricted number of HSP70 genes, and it may be that other family
476 members remain to be discovered (Clark et al. 2016). This will become more apparent with
477 the increase in NGS studies, as the krill work is demonstrating with six paralogous HSP70
478 gene family members identified to date (Casella et al. 2015). The environmental and the
479 evolutionary histories of organisms have a direct impact on the level of activation of the
480 transcription of Hsp70. We can distinguish between those organisms living in thermally
481 variable environments, such as the temperate pelagic zone and intertidal rocky shores, and
482 thermally stable environments, such as the tropical and polar zones (Tomanek 2010). Thus,
483 the HSP response will vary depending on the type of thermal environment: the more stable is
484 the environment, the weaker is the HSP response, with activation caused by a temperature
485 close to the thermal optimum of the species studied. In contrast, in a more variable
486 environment the HSP70 response will be more intense and occurs when the individual is
487 subjected to a temperature exceeding its thermal optimum and also across a wider range of

488 temperatures. In this context, the HSP response is a critical molecular mechanism in terms of
489 the thermo-tolerance capacity of individuals and contributes considerably to survival in case
490 of thermal stress. Animals exposed for several million years to varying thermal conditions
491 will have experienced positive selection for a very active heat shock response in contrast to
492 organisms living in stable environments, where there has been less evolutionary pressure on
493 this specialized biochemical pathway and up-regulation of HSP70 genes in response to a
494 stress is far more muted.

495 This study on the arcto-boreal krill *T. inermis*, besides improving the knowledge of the
496 physiology of the species, also takes a comparative approach aimed at highlighting and
497 understanding the strategies chosen in response to changing temperatures by phylogenetically
498 closely related species living in more or less variable cold environments.

499 Similar to the results from the Antarctic krill species, the structural designations of the
500 different genes as either inducible or constitutive did not fit with the experimental results. The
501 potentially constitutive isoforms were the most involved in the responses to heat shocks.
502 However, unlike the Southern Ocean species, the observed up-regulation in gene expression
503 generally occurred earlier in the time frame of the experiment and the levels were more
504 elevated. This is particularly the case when these results are compared to those of *E.*
505 *crystallorophias*, which is the most sensitive austral species examined to date using exactly
506 the same regime of thermal shocks, whereas *E. superba* remained largely unreactive. This last
507 result was particularly interesting because the two species *T. inermis* and *E. superba* exhibited
508 similar capacities of thermal tolerance, according to the values of the corrected CT₅₀, i.e. both
509 can cope with a temperature increase of around 16°C. It seems clear that the strategies
510 adopted by both *T. inermis* and *E. superba*, and to a lesser extent *E. crystallorophias*, are
511 different and related to the characteristics of the environments in which they live. The
512 constitutive forms were particularly involved in these responses. As observed in *E.*
513 *crystallorophias*, an up-regulation of both isoforms (A and B), which were also the most
514 represented quantitatively in terms of mRNA, represents an effective strategy for
515 economically responding to thermal stress. Indeed, the existence of a significant basal level of
516 these isoforms assures a rapid response to stress by allowing immediate translation of a high
517 amount of protein. Although in *T. inermis* only the B isoform was found, its behavior was
518 very similar to that of the isoforms of *E. crystallorophias*. The duplication of these isoforms
519 in *Euphausia* could constitute a significant advantage in response to either a heat or cold
520 shock. The hypothesis that ectothermic animals living in cold environments favor the
521 accumulation of Hsps to correct the damage directly linked to the problems of protein folding

522 at low temperatures could be an explanation for this physiological behavior (Place and
523 Hofmann 2005). A supplementary argument that might support this hypothesis, is that
524 Hsp70A mRNA amounts were 10 times less in *E. superba* fished in the warmer waters of
525 South Georgia than in animals from the East coast living at around 0°C (Tremblay et al.
526 submitted). Thus, the lack or the delay of gene expression could find its origin in the constant
527 presence of large amounts of Hsp proteins in animals, quantities that would be sufficient to
528 manage the immediate damages caused by a moderate or an acute increase of temperature
529 early on. When the shock is prolonged and quantities and/or degradation of available Hsp70s
530 become critical to correct cell damage caused by a thermal shock, the available mRNAs could
531 be recruited via translation and at that moment new mRNAs would be transcribed. As a
532 consequence, the higher the amounts of Hsp as protein or mRNA within the cell under normal
533 conditions, the more delayed the heat shock response would be. Thus highlighting the
534 importance of the numbers of copies available of the A and B forms. Therefore, monitoring
535 quantitative changes in translated Hsp70 in response to thermal shock will be the next step
536 towards a more complete understanding of response strategies among different species of
537 krill.

538 **Conclusions**

539 In the current study, we found that the 6°C experimental temperature was sufficient to
540 induce gene expression of the structurally defined constitutive *Hsp70* isoform. Up-regulation
541 of expression occurred during direct heat exposure as well as during recovery, thus
542 contradicting the structural definition of this isoform as “constitutive”. In addition, the high
543 level of gene expression during recovery (e.g. up to 25-45 fold expression in *Hsp70B* and *D*
544 respectively compared to the control) was indicative of intense molecular repair activities
545 taking place, even at this relatively low temperature. Isoforms C1 or C2, which are defined
546 structurally as inducible, showed no significant response at either 3 or 6 hours after shock or
547 during the recovery phase but in fact showed a reduction in available mRNAs.

548 The expression kinetics of these different isoforms are not similar to the response
549 expected in a temperate species. They are also different from the observed responses in other
550 species of krill from other cold environments such as Antarctica (Casella et al. 2015).
551 However, comparing the kinetics of expression between the polar species also revealed
552 similarities between constitutive isoform behaviors. Thus, we developed the hypothesis that
553 maintenance of a high constitutive protein level is associated with a large stock of mRNAs
554 and that this organization enables an instant or continuous response to heat stress. This may

555 be a primitive response to life in the cold, but also alter the threshold of transcriptional
556 induction of new mRNAs, as these are costly to produce. On this basis, *T. inermis* could be
557 considered as an intermediate type between very cold and stable environments and temperate
558 conditions.

559 However, even if the CT₅₀ of *T. inermis* was around 20°C, the current findings imply that
560 this species experienced molecular damage during exposure at 6-10°C, which is even lower
561 than their thermal respiratory limit at 12°C as found by the Arrhenius breakpoint temperature.
562 Therefore, the long-term thermal limit of overall species performance may be < 12°C at least
563 under experimental conditions and will almost certainly be lower under chronic temperature
564 challenges (Peck et al. 2009).

565 In order to more accurately predict the warming effects on the overall performance and
566 population persistence of the arcto-boreal *T. inermis*, further research is needed. The data
567 presented here are preliminary, but provide potential directions for further investigations.

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703

704 **Legends**

705 Figure 1: Curves representing the loss of mobility of krill populations subjected to gradual
706 temperature increases ($0.1.\text{min}^{-1}$) (*E. crystallorophias* n=130; *E. superba* n=43 (Cascella et
707 al., 2015); *T. inermis* n=41). CT_{50} is the temperature at which a 50% loss mobile animal was
708 reached.

709

710 Figure 2: Alignment of six Hsp70 isoforms from *T. inermis*. B, E, F isoforms represent the
711 potential constitutive forms, C1 and C2 the potential inducible ones and D the mitochondrial.
712 In red: Hsp70 diagnostic motifs; in black: possible glycosylation sites; in green: hydrophobic
713 linker between Nucleotide Binding Domain and Substrate Binding Domain. Alignment was
714 realised with CLC MainWorkbench 7.

715

716 Figure 3: Phylogeny of the Hsp70 family including Grp78 in Eucrustacea based on a
717 Bayesian analysis of the amino acid data set. Numbers above branches are posterior
718 probabilities. *T. inermis* isoforms are in bold. Figure was created with FigTree v1.3.1.

719

720 Figure 4: Arrhenius plot of normalized respiration rates of adult *T. inermis* (n=4-40) in
721 relation to experimental temperatures. Lines show linear regressions. ABT = Arrhenius
722 breakpoint temperature defined by a significant change in slope. Values are given as means \pm
723 SEM, n = number of individuals used in the temperature experiments. * Significant difference
724 to 4°C control temperature.

725

726 Figure 5: Mean normalized expression ratios of five *Hsp70* isoforms in the muscle tissue of
727 adult *T. inermis* (n=6-10) during 3 h (A) and 6 h (B) continuous heat shock at 6 or 10°C
728 followed by 6 h of recovery at control temperature (4°C; shaded). Values relate to the control
729 group (specimens continuously kept at 4°C). *: Significant MNE difference to 4°C control
730 temperature evaluated with a Kruskal-Wallis test. Figure was created using GraphPad Prism
731 6.0h.

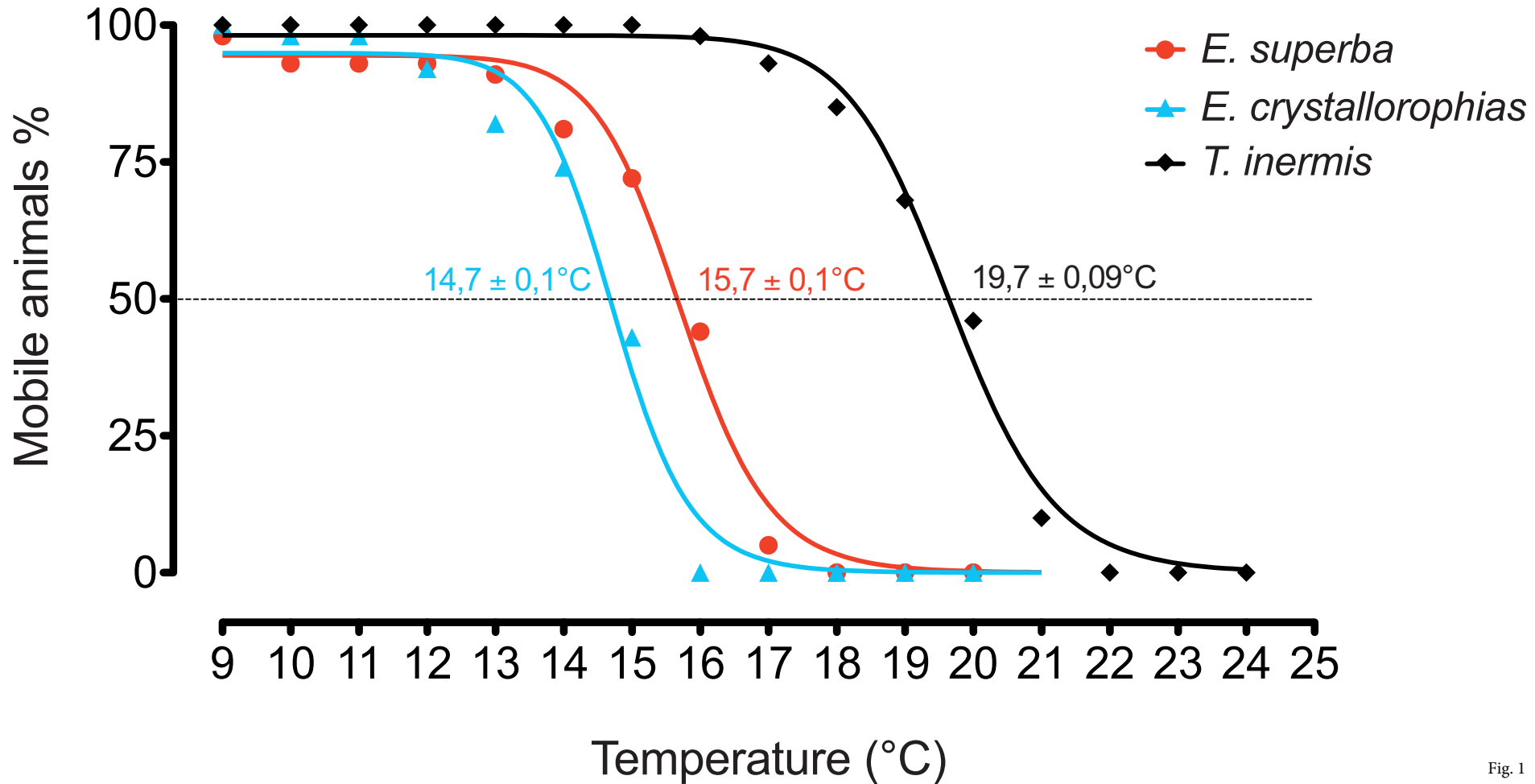


Fig. 1

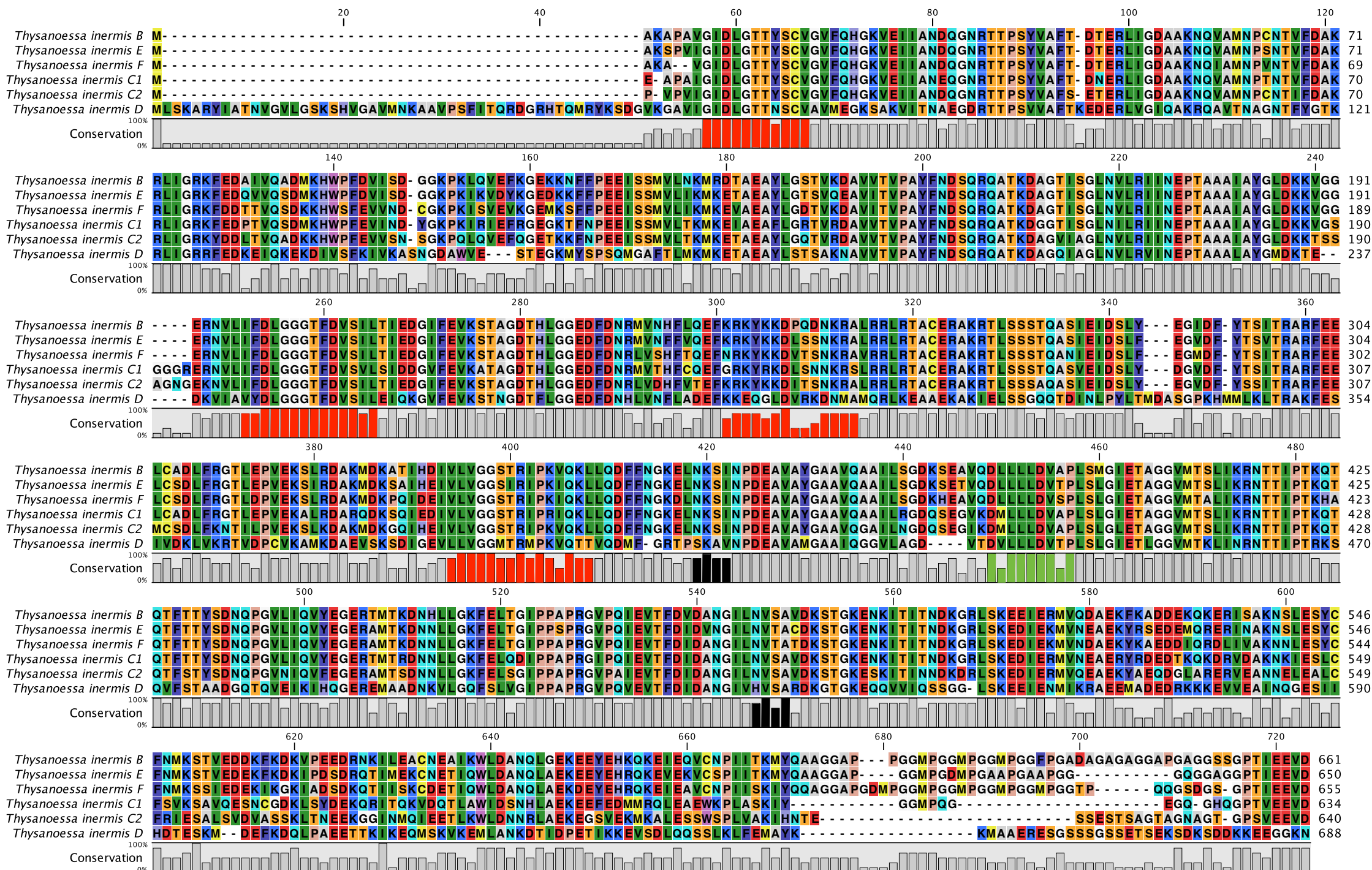


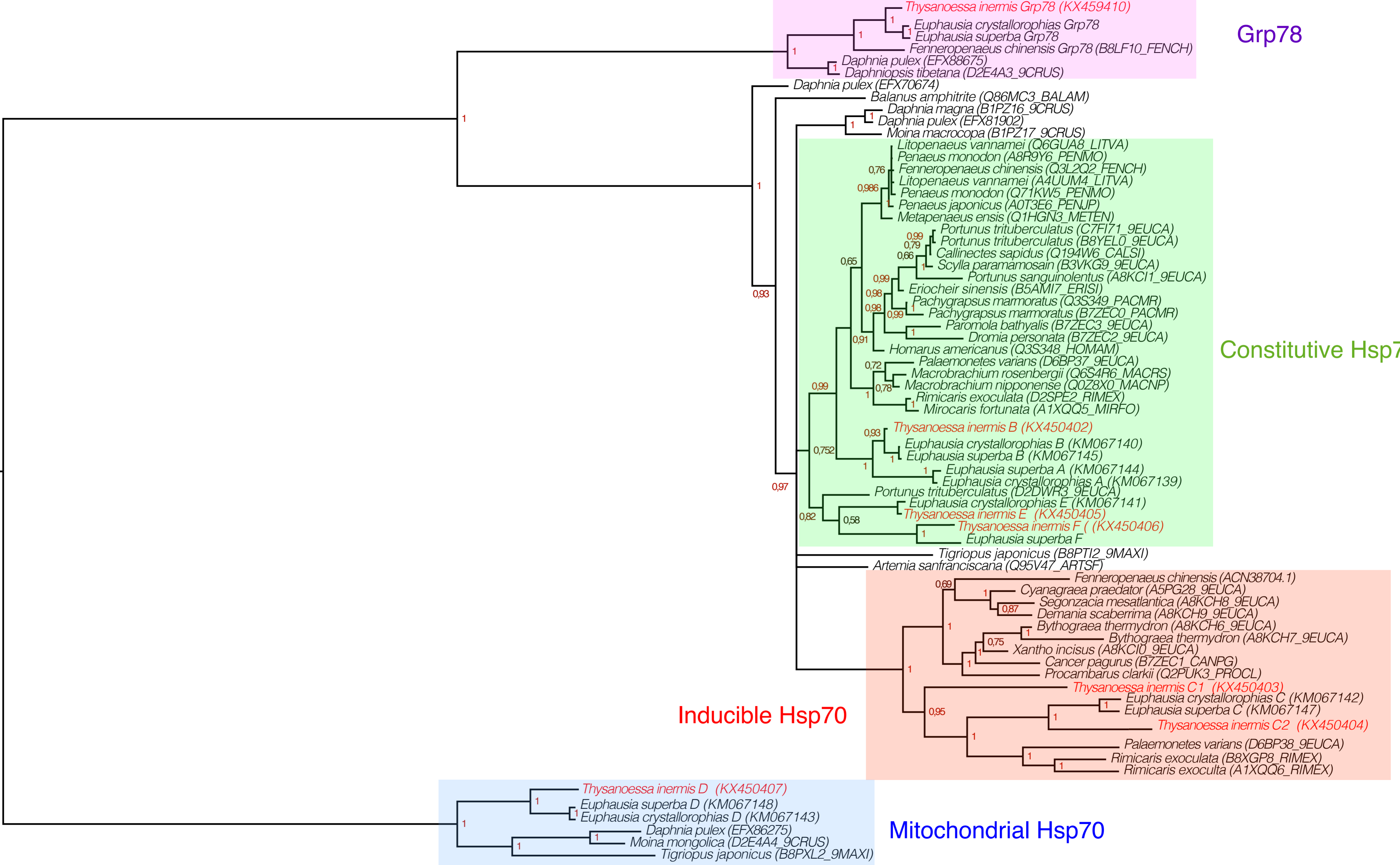
Fig. 2

Grp78

Constitutive Hsp70

Inducible Hsp70

Mitochondrial Hsp70



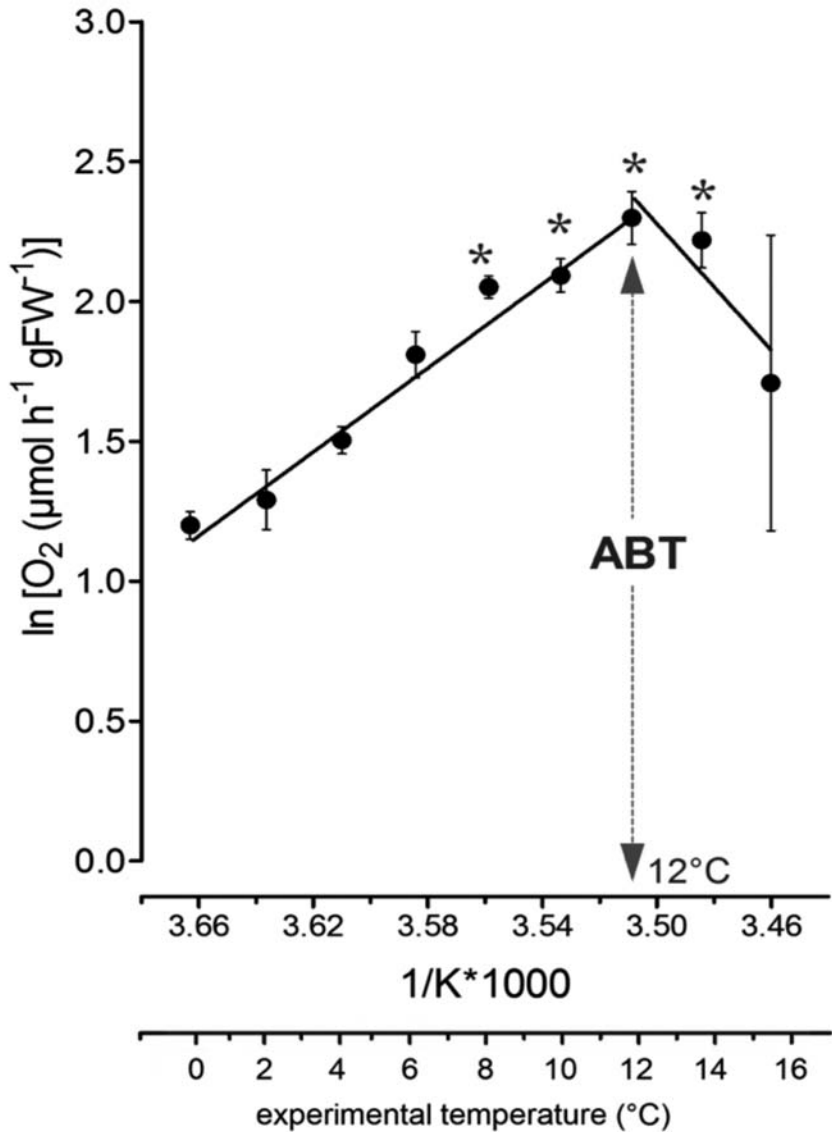


Fig. 4

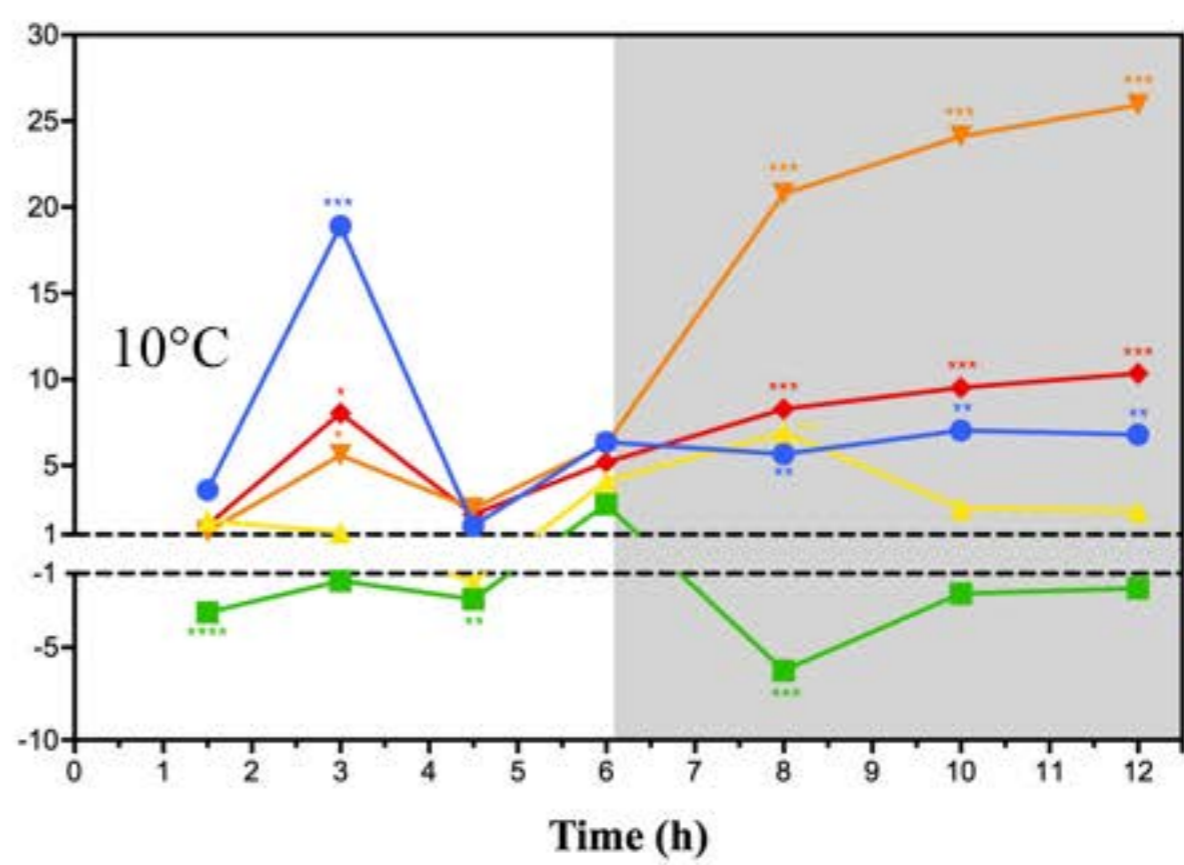
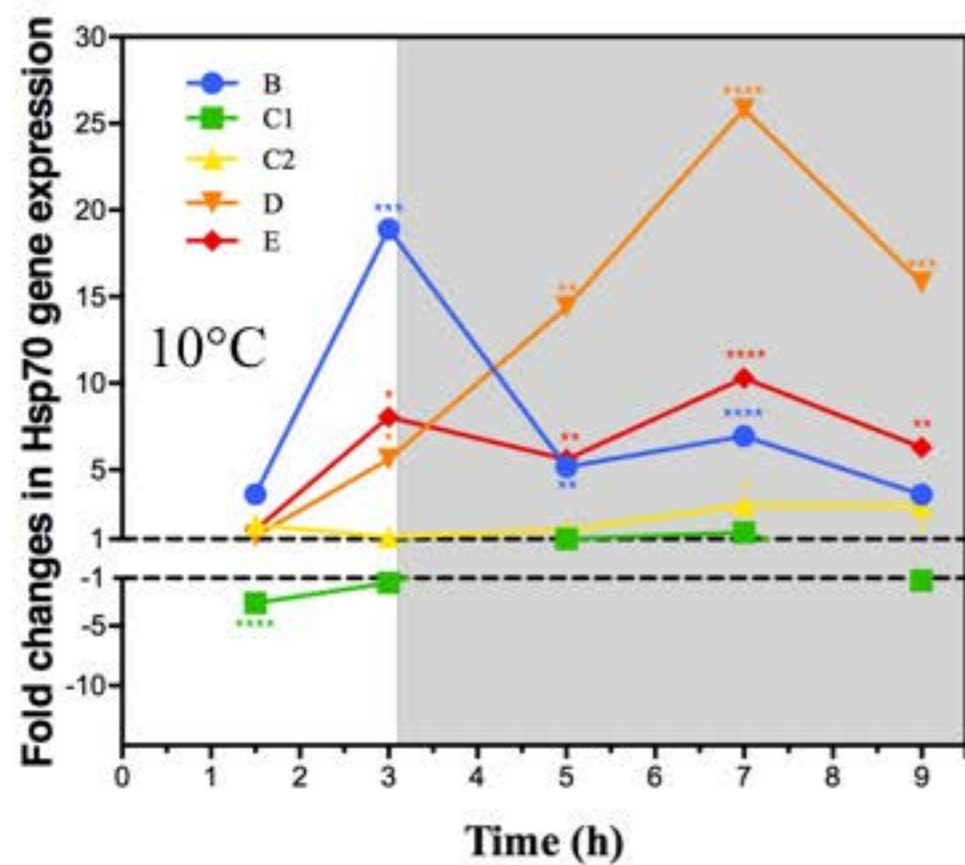
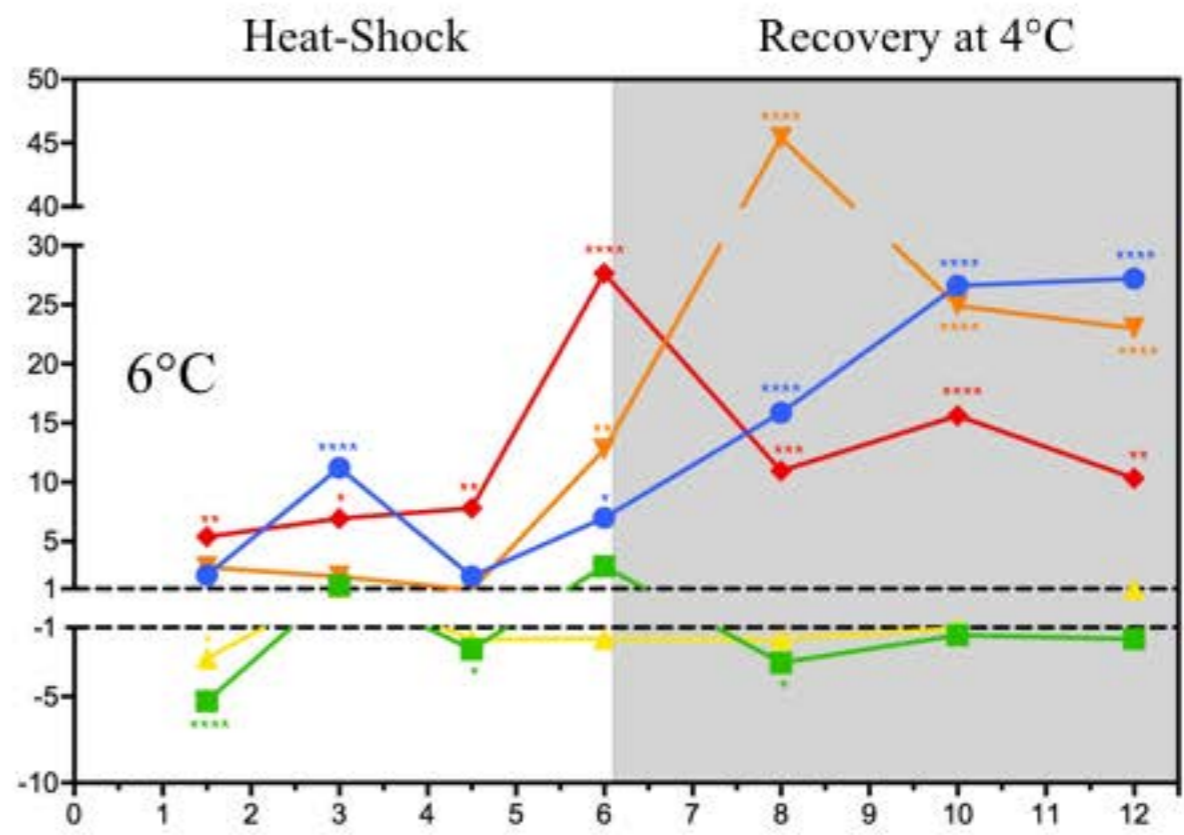
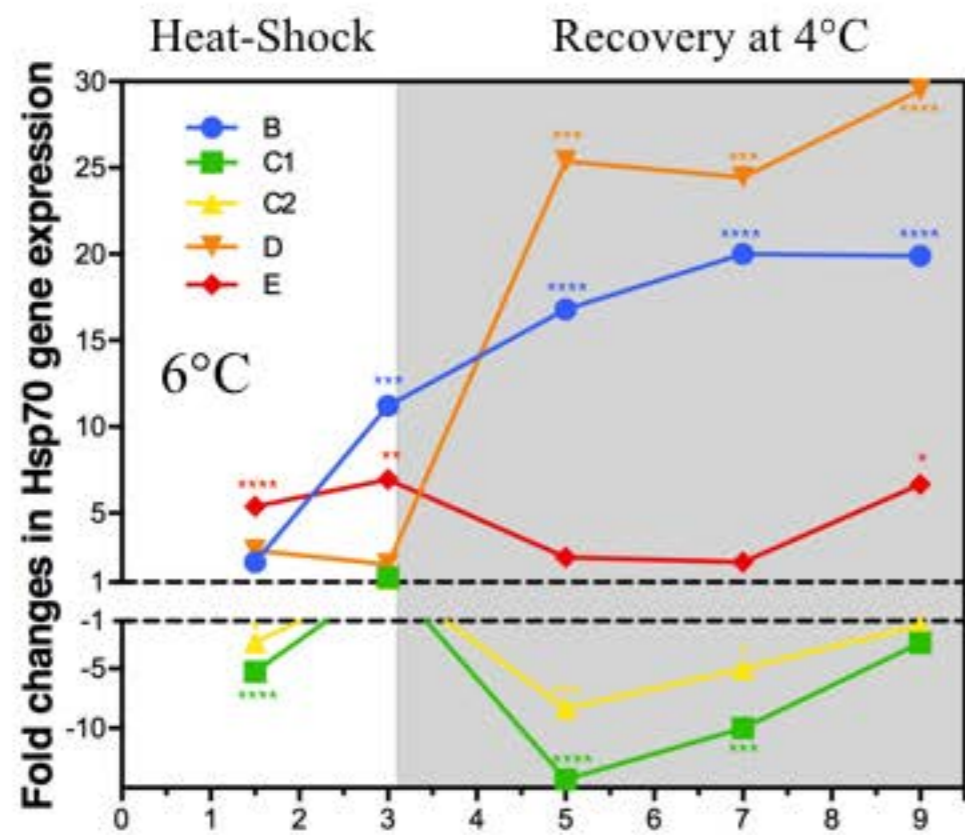


Fig. 5