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## Antarctic krill (*Euphausia superba*) in a warming ocean: thermotolerance and deciphering Hsp70 responses

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1           **Antarctic krill (*Euphausia superba*) in a warming ocean:**  
2           **thermotolerance and deciphering *Hsp70* responses**

3  
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18  
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## 32 **Abstract**

33

34 The Antarctic krill, *Euphausia superba*, is a Southern Ocean endemic species of proven  
35 ecological importance to the region. In the context of predicted global warming, it is  
36 particularly important to understand how classic biomarkers of heat stress function in this  
37 species. In this respect, Hsp70s are acknowledged as good candidates. However, previous  
38 studies of expression kinetics have not been able to demonstrate significant upregulation of  
39 these genes in response to heat shocks at 3°C and 6°C for 3 and 6 hours. The current work  
40 complements these previous results and broadens the prospects for the use of Hsp70s as a  
41 relevant marker of thermal shock in this krill species. New experiments demonstrate that  
42 induction of Hsp70 isoforms was not detected during exposure to heat shock, but increased  
43 expression was observed after several hours of recovery. To complete the analysis of the  
44 expression kinetics of the different isoforms, experiments were carried out over short time  
45 scales (1 and 2 hours at 3°C and 6°C) as well as at higher temperatures (9°C, 12°C and 15°C  
46 for 3 hours), without any significant response. A 6-week monitoring of animals at 3°C  
47 showed that the time factor is decisive in the establishment of the response.  $CT_{max}$   
48 experiments with incremental times of 1°C per day or 1°C every three days have shown a  
49 particularly high resilience of the animals. The demonstration of the abundance of Hsp70s  
50 present before thermal stress in various species of krill, as well as in specimens of *E. superba*  
51 of various origins, showed that the delay in the response in expression could be related to the  
52 high constitutive levels of Hsp70 available before the stress experiments. The alternative  
53 labelling of the two main isoforms of Hsp70 according to the origin of the animals allowed  
54 hypotheses to be put forward on the functioning of thermoregulation in Antarctic krill as well  
55 as ice krill.

56

## 57 **Introduction**

58 Global Warming constitutes a Grand Challenge for Biology as defined by Schwenk et al.  
59 (Schwenk et al. 2009) and Denny and Helmuth (Denny and Helmuth 2009). It represents a  
60 major, unsolved problem that requires a significant improvement in capability or a  
61 fundamental change in perspective to solve. In this respect, the pivotal role of physiology has  
62 been highlighted in confronting the complex problems posed by global climatic change, and  
63 the need to strive toward a common focus, the whole organism: “*It is physiology - our*  
64 *understanding of how individual organisms function and interact with their environment -*  
65 *that presents the largest challenge. Without a better mechanistic understanding of how plants*  
66 *and animals work, we can never be assured of an accurate warning of what lies ahead for life*  
67 *on earth*” (Denny and Helmuth 2009).

68 To fit with this strategy, the choice of the biological model is extremely important. The  
69 Southern Ocean is an important breeding and/or foraging location for a wide range of  
70 charismatic megafauna such as whales, seals, penguins and other sea birds including  
71 albatrosses. Whilst these species fascinate the public, they represent the apex of a complex  
72 food chain, the keystone species of which is the Antarctic krill *Euphausia superba*  
73 (Trivelpiece et al. 2011). These shrimp-like crustaceans are not only a major prey item for  
74 these animals, but are also significant consumers, grazing on the phytoplankton bloom in the  
75 austral summer and on algae under the sea ice in winter for the most southern ones. In a more  
76 global context, the Antarctic krill has been suggested to be the most abundant eukaryotic  
77 species in the World’s Oceans existing in large schools or swarms with densities that may be  
78 between 10,000 - 30,000 individual animals per square meter (Macaulay et al. 1984). In  
79 addition, it is an increasingly profitable fisheries resource. It is therefore essential to have  
80 maximum information on the thermal resilience of these animals in order to best manage this  
81 stock (Atkinson et al. 2004; Everson 2000; Flores et al. 2012; Schiermeier 2010). Given the  
82 central role of this key species in the Southern Ocean ecosystem, detailed physiological and  
83 molecular studies are essential to understand this species and its responses to a changing  
84 environment.

85 This work continues a previous study focused on the capacity of Antarctic krill to respond to  
86 a rise in environmental temperature (Cascella et al. 2015). The Cascella study included the  
87  $CT_{max}$  values of two krill species from the Antarctic zone *Euphausia superba* and *Euphausia*  
88 *crystallorophias*, as well as the first measurements of the expression of different Hsp70

89 isoforms in response to thermal shocks of 3 and 6 hours to 3°C and 6 °C. These first results  
90 showed, especially in *E. superba*, a very weak response in terms of Hsp70 expression during  
91 the shock at these different temperatures. Furthermore, a second study on the arcto-boreal  
92 krill *Thysanoessa inermis*, which lives in warmer waters and should be less stenothermal, has  
93 shown a similar thermal resilience ( $CT_{max}$ ) to the Antarctic species, but with different  
94 expression kinetics, especially in terms of isoform reactivity (Huenerlage et al. 2016).

95 In the current study, further experiments were performed to interrogate in more detail the  
96 question of whether *E. superba* is capable of an HSP response. Measurements were  
97 performed on the expression of the different Hsp70 isoforms in the recovery period (post-  
98 shock), but also after short shocks of 1 and 2 hours and more intense thermal shocks at 9°C,  
99 12°C and 15°C. In addition, a study of the expression in response to a long shock at 3 °C for 6  
100 weeks was conducted in an aquarium under controlled conditions. Finally, organismal heat  
101 tolerance was assayed using a slower rate of warming than prior studies to provide an  
102 ecologically relevant measure of tolerance.

## 103 **Material and Methods**

104

### 105 **Animal collection**

106 *E. superba* were obtained from different sources, depending on the experiment (see Figure 1).  
107 *E. superba* for acute trials at 3°C and 6°C were fished off the French base Dumont d'Urville  
108 (DDU) in Terre Adélie just beyond the continental plateau (65.30°S, 139.57°E). Fishing was  
109 carried out using an Isaacs-Kidd midwater trawl (IKMT) from the ship « L'Astrolabe » at a  
110 maximum speed of 2 knots for 10-15 minutes. The sampling depth was determined by sonar  
111 observations of swarms, taking samples from those with a depth of between 20 and 50 meters.  
112 After fishing, the animals were moved to a refrigerated flow-through tank on board, which  
113 was continuously supplied with seawater at -0.5 - +0.5°C. Animals were acclimated for 24  
114 hours to aquarium conditions prior to experimentation to minimize holding stress. A  
115 circulation pump was used to set up a vertical current maintaining the animals in the water  
116 column.

117 A second series of acute thermal trials and longer thermal shocks were performed in the  
118 temperature-controlled aquariums of the Australian Antarctic Division in Kingston  
119 (Tasmania) (Kawaguchi et al. 2010). Animals used for these experiments were fished at

120 64.11°S, 111.12°E and kept in aquaria for 8 months before being challenged with heat.  
121 Samples of *E. superba* from western Antarctica (62.29°S, 139.59E) were kindly provided by  
122 Drs. Juan Höfer (IDEAL, Chile) and Marcelo Gonzalez-Aravena (INACH, Chile) for the  
123 Western blots.

124 This project (IPEV-1039) was approved by IPEV (Institut Paul Emile Victor, the French  
125 Polar Institute) review committee and was declared to, and approved by, the “Terres  
126 Australes et Antarctiques Françaises” in 2009 according the Annex I of the Madrid Protocol  
127 and the French Decret No 2005-403. No endangered or protected species were used.

## 128 **Experiments**

### 129 **Thermal shocks at 3°C and 6°C (onboard Astrolabe)**

130 After around 24 hours in the main aquarium, the most mobile animals were selected and put  
131 into two aquaria containing seawater at the same temperature as the main stock aquarium.  
132 These aquaria were placed within a larger tank containing fresh water. A copper coil linked to  
133 a thermostat and pump was added to the system. The coil was used to pump hot water around  
134 the system to warm up the tank water for experiments. A circulating pump ensured that the  
135 warming was equally distributed around the tanks and air pumps were used to maintain the  
136 oxygen levels in the aquaria and also facilitate water mixing.

137 The protocol of the experiments is depicted in Figure 1A. For each thermal shock, animals  
138 from the same trawl were treated at the same time for 3 or 6 hours before being returned to  
139 0°C seawater for recovery. The animals were put directly into the warmed water. At the same  
140 time, a set of control animals from the main aquarium were snap frozen in liquid nitrogen.  
141 Animals having undergone heat shock at 3°C or 6 °C were sampled after 3 or/and 6 hours of  
142 shock and 2, 4, 6 hours of recovery (post shock (PS)) and deep-frozen. Each sampling  
143 comprised 10 animals.

### 144 **Acute shocks at 3°C and 6°C (Australian Antarctic Division)**

145 *E. superba* were directly transferred from the tank at 0°C to the tanks at 3°C and 6°C. Ten  
146 animals were collected after 1 and 2h and immediately snap frozen in liquid nitrogen and then  
147 kept at -80°C until required.

### 148 **Acute shocks at 9°C, 12°C and 15°C for 3 hours (Australian Antarctic Division)**

149 *E. superba* were directly transferred from the tank at 0°C to the tanks at 9°C, 12°C or 15°C.  
150 Ten animals were used at each temperature. Heat shocked animals were sampled after 3h and  
151 directly frozen in liquid nitrogen before storage at -80°C.

### 152 **Longer thermal trials at 3°C for 6 weeks (Australian Antarctic Division)**

153 120 *E. superba* were warmed from 0°C to 3 °C at 1°C/h. Ten animals were sampled at 3, 6,  
154 12, 24 hours and then, at the same time, every day for 2 days and finally every week until the  
155 sixth week. Animals were snap frozen and samples kept at -80°C. Ten more animals from the  
156 original tank were directly frozen and used as control (Fig 1B).

### 157 **CT<sub>max</sub> experiments**

158 CT<sub>max</sub> experiments were carried aboard the Astrolabe using increments of 1°C/10 min  
159 according to the protocol set out in Cascella et al. (Cascella et al. 2015). Additional CT<sub>max</sub>  
160 experiments were carried out at rates of 1°C per day and 1°C every three days. These  
161 experiments were carried out in the AAD aquaria in Kingston (Tasmania). Animals no longer  
162 responding to a tactile stimulus during this thermal trial were removed from the aquarium.  
163 The CT<sub>max</sub> was considered as the temperature at which 50% of experimental animals were  
164 still mobile. It was determined though the non-linear curve fitting option in JMP10 (SAS).  
165 The survival curve used was:  $Survival = c / (1 + (T/CT_{max})^b)$  where c is the plateau value before  
166 the sharp decrease, CT<sub>max</sub> the temperature at which 50% of mobile animals is reached, and b a  
167 sigmoidicity coefficient. The program explores the different values of these three parameters,  
168 and calculates a Chi-square value. While exploring the different parameter values, the  
169 program aims to minimize this Chi-square value and converges towards a value for each  
170 parameter (provided with a standard error). The numbers of animals used for these CT<sub>max</sub>  
171 experiments were 43, 30 and 30 for 1°C per 10 minutes, 1°C per day and 1°C per 3 days  
172 respectively.

### 173 **qPCR analysis**

174 mRNA levels of the Hsp70 isoforms were determined by quantitative RT-PCR amplification,  
175 on 6 to 10 individuals for each experimental condition according to the same protocol in the  
176 previous publication (Cascella et al. 2015). A dissociation curve was generated and PCR  
177 efficiency was estimated for each primer pair. All primer pairs tested generated a single peak  
178 in the dissociation curve and a PCR efficiency of 80 - 100%. Data were analysed with the  
179 LightCycler 480 software. The 18S gene was chosen as a reference gene using the



180 BestKeeper algorithm (Pfaffl et al. 2004) after testing EF1 $\alpha$ , 18S, RPL8, and GAPDH as  
181 potential normalizing housekeeping sequences. *Hsp70* expression was subsequently  
182 normalized to this reference. Differences of the mean normalized expression (MNE) of the  
183 five *Hsp70* isoform genes (A, B, C, D and E) over the time course of the experiment were  
184 analyzed using a non-parametric Kruskal–Wallis test and a Dunn’s multiple comparison test  
185 to determine significant differences between control and heat shocked samples. Relative gene  
186 expression (fold *Hsp70* expression) was calculated from the MNE of test specimens (heat  
187 shock at 3°C or 6°C for 3 or 6 hours and subsequent recovery at 0°C) divided by the MNE of  
188 control specimens (kept at 0°C control temperature).

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

### 191 **Western blot**

192 Samples of krill abdomens without their cuticle were ground in liquid nitrogen, and the  
193 powder was homogenized in 1 ml of extraction buffer (20 mM Tris/HCl, pH 7.5: 40  $\mu$ l  
194 protease inhibitor cocktail (Roche, France)). The homogenates were centrifuged at 10,000 g  
195 for 15 min at 4 °C. The extracted proteins were quantified in the supernatant with the Bio-Rad  
196 Protein Assay (Bio-Rad, Marnes-la-Coquette, France) using bovine serum albumin (Sigma)  
197 as standard. Proteins of the total supernatant were separated by sodium dodecyl sulfate–  
198 polyacrylamide gel electrophoresis (SDS–PAGE; 7.5% acrylamide: 0.3% bisacrylamide  
199 (w/v); 20  $\mu$ g protein well<sup>-1</sup>). After running the electrophoresis (15 min at 15 mA gel<sup>-1</sup>, 1 h  
200 at 20 mA gel<sup>-1</sup>), the bands were stained with Coomassie Blue. Protein molecular mass  
201 standards from 10 kDa to 250 kDa (All Blue, Bio-Rad) were used to evaluate the apparent  $M_r$   
202 of the separated bands.

203 For western blotting, the proteins were transferred from the SDS–PAGE gel  
204 (20  $\mu$ g protein well<sup>-1</sup>) to a nitrocellulose membrane by semi-dry blotting at 25 mA for 2 h  
205 (Trans-Blot semi-dry cell; Bio-Rad). Membranes were blocked in milk/BSA/Tris-buffered  
206 saline pH 7.5 (TBST: Tris Buffered Saline with Tween 20) (2.5% w/v each) for 1h, rinsed in  
207 TBST and incubated overnight with a polyclonal antibody (1/1000 Mouse anti-chicken  
208 HSC70/HSP70 monoclonal antibody; Enzo Life Sciences, Lausen, Switzerland) at room  
209 temperature (Ravaux et al. 2003). Subsequently, a 10 min washing step with TBST with 2.5%  
210 milk pH 7.5 was repeated four times, and the membranes were incubated with a polyclonal  
211 secondary antibody coupled to horseradish peroxidase (HRP) (1/3000 donkey anti-mouse

212 IgG-HRP: sc-2314, Santa Cruz Biotechnology Inc.) for 1 h at room temperature. After  
213 another 4×10 min washing step with TBST, the antibody complex was detected using the  
214 Luminol reagent substrate (SuperSignal West Femto Maximum Sensitivity Substrate, Thermo  
215 Scientific, Redford, USA) incubated at room temperature for 1 min and read with a Fusion  
216 FX (Vilber Lourmat, Marne la Vallée, France).

217

## 218 **Results and discussion**

219

### 220 **Upper critical thermal limits vary according temperature increment rate**

221 As a preliminary analysis to understand the thermal resilience of *E. superba* in the Southern  
222 Ocean, short term acute exposures were first undertaken, using a temperature increase of  
223  $0.1^{\circ}\text{C}/\text{min}$  (Cascella et al. 2015). The interpretation of the  $\text{CT}_{\text{max}}$  curve of *E. superba* gave an  
224 unexpectedly high value of  $15.8 \pm 0.1^{\circ}\text{C}$  for a stenothermal Antarctic ectotherm (Peck et al.  
225 2009). As the thermal shocks applied in these first sets of experiments were relatively short  
226 and represented very rapid temperature increases, thermal shocks with longer increment times  
227 were conducted to confirm or refute the apparent thermal resistance highlighted by these  
228 previous experiments. Indeed, Peck et al. showed that  $\text{CT}_{\text{max}}$  varied with the rate of  
229 experimental warming, more precisely  $\text{CT}_{\text{max}}$  declined at slower rates of warming (Peck et al.  
230 2009; Peck et al. 2014). Thus, the time elapsed between two temperature increments of  $1^{\circ}\text{C}$   
231 was significantly increased since the curves presented were obtained for intervals of 1 and 3  
232 days (Figure 2).

233 Observed  $\text{CT}_{\text{max}}$  values were  $14.7 \pm 0.1^{\circ}\text{C}$  and  $13.9 \pm 0.03^{\circ}\text{C}$  for increments of  $1^{\circ}\text{C}$  per 1 and  
234 3 days respectively. These values appear particularly high compared to what has been  
235 regularly observed in other polar invertebrates (Peck et al. 2014). More especially, the small  
236 difference between the  $\text{CT}_{\text{max}}$  as a function of the time between each temperature increase  
237 was surprising. Overall, the decrease in  $\text{CT}_{\text{max}}$  was approximately  $1^{\circ}\text{C}$  between the different  
238 rate of change experiments. This small decrease highlights a particularly high temperature  
239 resilience. This result makes sense when comparing these values with those found with  
240 another polar eucarid, the shrimp *Chorismus antarcticus*, a species phylogenetically closer than  
241 most of the Antarctic invertebrates studied so far, with  $\text{CT}_{\text{max}}$  values of  $11.6^{\circ}\text{C}$  and  $6.7^{\circ}\text{C}$  for  
242 increments of  $1^{\circ}\text{C}$  every 10 minutes or every day respectively (personal and unpublished  
243 data). In this example, the  $\text{CT}_{\text{max}}$  for a  $1^{\circ}\text{C}/10$  min was already much lower than that of *E.*  
244 *superba* but drops significantly with the extension of the warming interval. These differences  
245 are certainly partially attributable to the differences in living environment, which is pelagic  
246 beyond the continental shelf for *E. superba* versus benthic on the continental shelf for *C.*  
247 *antarcticus* (Peck et al. 2009).

### 248 **Kinetics of Hsp70 expression**

249 *Post-shock kinetics*

250 In a previous publication (Cascella et al. 2015), the expression kinetics of five *E. superba*  
251 *Hsp70* isoforms (A, B, C, D, E) were analyzed in response to moderate thermal shocks at 3°C  
252 or 6°C for 3 or 6 hours. Results showed that, this response in Antarctic krill was very weak  
253 when compared with the fold changes observed in temperate species (Feder and Hofmann  
254 1999; Ravaux et al. 2012). In this study, the initial heat shock experiments of Cascella et al  
255 (2015) were expanded to include a recovery period at 0°C (Figure 3). According their  
256 structural characteristics and phylogenetic position, the A, B and E isoforms were most  
257 similar to the traditional constitutive form gene cluster. The C isoform is most similar to  
258 inducible isoforms in other species. These four isoforms are cytoplasmic while the D isoform  
259 is most similar to the group of mitochondrial Hsp70s (Baringou et al. 2016; Cascella et al.  
260 2015; Huenerlage et al. 2016).

261 The rapid transition from 0°C to 3°C did not invoke an up-regulation of *Hsp70* in *E. superba*  
262 (Figure 3a). Actually, there was a general trend for a reduction in Hsp70 gene expression after  
263 6 hours compared with the controls (Figure 3b). However, if the up-regulation of expression  
264 recorded during the actual heat shock itself was weak, the analyses of post-shock showed  
265 much higher levels of expression, which increased with the length of the initial shock. The  
266 most responsive isoform was the E form, both in terms of speed of response and intensity.  
267 Nevertheless, even though this form was the only one showing significant up-regulation  
268 during the recovery phase after 3 hours of shock (Figure 3a), the duration of the shock greatly  
269 influenced the amplitude of the responses of the other isoforms. The expression kinetics were  
270 clearly distinct according to the Hsp70 isoforms. However, similarly to the results in Cascella  
271 et al. (2015), there was no correlation between the expression levels and the category of  
272 isoform group to which they belonged, i.e. the group of constitutive forms (theoretically no  
273 up-regulation to heat shock) or the group of inducible forms (theoretically strong up-  
274 regulation to heat shock).

275 It is also clear that, if the duration of the shock influences the importance of the expression,  
276 the intensity of the shock impacts this response as well. Indeed, a shock of 3h at 6°C produced  
277 a larger response for isoforms A and B than a shock of identical duration at 3°C, with a  
278 maximum observed at 6h post-shock. This response was measured paradoxically only for  
279 these so-called constitutive forms (Figure 3c). However, the up-regulation patterns are very  
280 different for 6 hours at 6°C, where the response is far more muted with a trend in overall

281 decreases in the responses recorded so far (Figure 3d). This lack of up-regulation may  
282 indicate that the animal needs more time for recovery or has reached its limits and no longer  
283 has the ability to compensate for variation in the environment.

284 Be that as it may, even if the up-regulation in Hsp70 in response to thermal shock did not  
285 reach the values observed in other ectotherms, especially from temperate regions, the  
286 recovery period values were much higher than those measured during the shock itself. *E.*  
287 *superba* is therefore unquestionably capable of responding to a thermal shock via up-  
288 regulation of its Hsp70s. The very low level or the absence of response observed during the  
289 heat shock is curious. Several reasons could explain the values observed: i) the timing of first  
290 samplings were too late and did not allow for a very rapid and early response, within the first  
291 three hours ii) the applied temperatures were too low to generate a response iii) Antarctic krill  
292 has a large constitutive level of Hsp70 proteins, which it can use as an available pool to cope  
293 with the molecular perturbations induced by the increase in temperature, thus explaining the  
294 late up-regulation of mRNAs. Each of these hypotheses was tested in the following  
295 experiments.

#### 296 ***Short heat shock kinetics***

297 In order to follow the kinetics of expression over shorter times, a new series of heat shock  
298 experiments (+3°C and +6°C) were conducted according to an identical protocol but with  
299 sampling points at 1 and 2 hours (Figure 4).

300 No significant up-regulation was observed except for E isoform after 2 hours of heat shock.  
301 The values obtained were similar to the trends observed with 3 hours of heat shock. However,  
302 it is interesting to note significant down-regulation of expression after 1h for the A isoform  
303 and after 1 and 2 hours for the B isoform. The results are similar for both temperatures. This  
304 decrease in expression could be attributed to a massive recruitment of mRNAs, originally  
305 present before the heat shock, for protein synthesis. Much of the response would then partly  
306 consist of replacing the mRNAs involved in translation, when the shock remains moderate in  
307 duration and intensity. On the other hand, the potentially inducible form C does not show any  
308 significant expression variation. Again, as demonstrated throughout the heat shocks, the so-  
309 called constitutive forms are the most involved in the response of *E. superba* to temperature.

310 In summary, the hypothesis of an upregulation in Hsp70s prior to the original 3-hour  
311 sampling period was not validated.

312 ***Heat shock kinetics***

313 In order to test the hypothesis that the weak response observed after thermal shocks at 3°C  
314 and 6°C was, or was not, related to the fact that the intensity of these shocks was insufficient,  
315 the animals were immersed for 3 hours in water at 9°C, 12°C and 15°C, then expression of  
316 *Hsp70s* were measured (Figure 5).

317 As a first observation, none of the animals immersed in water at 15°C survived the  
318 experiment and only half of the individuals resisted for 3 hours in water at 12°C, thus the  
319 12°C results represent a resisting population, which may explain why expression levels are  
320 slightly higher at 12°C for some isoforms. This observation highlights the importance of the  
321 implementation of the thermal shock. These animals were directly immersed in water at the  
322 shock temperature, with no gradual warming. The gradual raising of temperature is an  
323 essential component in the ability of animals to manage the increase in environmental  
324 temperature, as occurs with potential acclimation. Indeed, the  $CT_{max}$  curves (Figure 2)  
325 demonstrate for example that no loss was recorded at the temperature of 12°C with  
326 increments of 1°C every 10 minutes or even 1°C every day.

327 As second observation, no significant up-regulation was recorded at these temperatures.  
328 However, as observed during the acute heat shocks, there was a down-regulation of isoforms  
329 forms A and B at 9°C only. This decrease in the amount of mRNAs encoding the constitutive  
330 *Hsp70s* appears to be a first step in the response process. It was present at 3°C and 6°C but  
331 seems to extend to 9°C before a trend of up-regulation as observed during the recovery period  
332 (Figure 3). Finally, when the length of the heat shock is extended, there might be a  
333 neosynthesis of mRNA coding for these chaperones following the disappearance of feedback  
334 inhibition generated by native *Hsp70* proteins. Thus, the absence of up-regulation during heat  
335 shocks and its appearance during post-shock recovery periods would not be linked to an  
336 absence of response or a potential recovery of animals but rather to a delayed response related  
337 to a native abundance of functional proteins, which is supported by large constitutive  
338 quantities of mRNAs of A and B isoforms in particular. These reflections lead us to postulate  
339 that *E. superba* has naturally high quantities of HSP70 proteins, but to date, HSP70  
340 abundance in this species has only been evaluated at the mRNA level.

341 ***Long-term heat shock***

342 The objective of this experiment was to estimate the ability of *E. superba* to acclimate to 3°C.

343 The aim was to follow the expression of the different isoforms during this process, which  
344 would correspond to a scenario in agreement with a future global warming or, simply to a  
345 sudden change of geographical zone or of water layer. The scenario of global warming is  
346 obviously difficult to achieve since it implies a gradual increase over several years. The  
347 second scenario is more likely, both because of the longevity of the members of the species  
348 and their migrations through geographical areas with waters of different temperatures.

349 The first notable result was the total absence of mortality in this experiment. Clearly, on this  
350 sole criterion of survival, the transition to 3°C seemed to have no impact on the animals and  
351 they appear to have acclimated.

352 Secondly, in view of the previous results, the implementation of a long heat shock was of  
353 great interest. Indeed, it should allow us to determine unequivocally if the responses observed  
354 during the post-shock recovery experiments could be due to a return to the original conditions  
355 and was therefore due to a recovery or rather to a belated response as suggested above. The  
356 isoform D was not evaluated because of the absence of a measurable signal in the majority of  
357 sampled times.

358 In general, the expression levels did not remain constant for the isoforms (Figure 6). Their  
359 kinetics of expression showed peaks at different times highlighting the presence of *Hsp70*  
360 regulation beyond 6 hours and therefore their activity of repairing cellular damage caused by  
361 chronic temperature challenge. The most important expression variations were recorded from  
362 12-hours onwards. It is therefore clear that post-shock conditions are not solely responsible  
363 for increasing the HSP70 response. The time factor is also fundamental.

364 The different isoforms showed very different kinetics in amplitude and chronology. The E  
365 isoform remained the most reactive and confirmed the data obtained during short heat shocks.  
366 The C isoform, which is structurally affiliated to inducible Hsp70s, showed a significant  
367 increase in expression after 12 hours, which is therefore rather late compared to 3°C animals  
368 measured after acute shocks and post-shock recovery. Similarly, the so-called constitutive  
369 isoforms, A and B, underwent a significant decrease on day 1 before recording an up-  
370 regulation on day 3. The comparison of the kinetics of expression of these isoforms also  
371 reveals variations in response between the animals in recovery and those maintained under  
372 extended heat shock conditions. The latter showed significant down-regulation, especially  
373 around day 1 probably related to mRNA recruitment in these chronic stress conditions,

374 followed by up-regulation. Overall, the patterns of up-regulation were delayed, compared  
375 with acute heat shocks, probably because the isoforms were originally present in large  
376 quantities, either as protein or mRNA.

377 It is clear that the kinetics of expression vary on the hourly scale at least during the first day.  
378 This observation is also valid on a day-to-day basis with respect to the differences recorded  
379 during the first three days of heat shock, although it is perfectly reasonable to think that a  
380 daily sampling smoothed the data and obscured probable short-term variations. This reflection  
381 is even truer at the scale of the week when the probability that there was a synchronism  
382 between the variations of expression and the sampling time remains low. Nevertheless, the  
383 six-week heat shock expression data appeared to show a general decrease until a slight down-  
384 regulation was observed in the last week. This raises the question as to whether this overall  
385 decrease in expression was due to a metabolic decline linked to the duration of the heat shock  
386 or to a process of acclimatization that is gradually taking place, which was not possible during  
387 the course of this experiment. *A posteriori*, it would be interesting in the future to finalize this  
388 study by a daily, or even better, an hourly monitoring of isoform expressions.

### 389 **What about constitutive HSP70 quantities?**

390 PCR monitoring of RNA expression provides information on the impact of a heat shock, but  
391 this approach, although powerful, considers only a part of the metabolic cascade involved. It  
392 is obvious that monitoring the production of proteins in response to a heat shock would also  
393 be useful as proteins are the end production of transcription/translation. Unfortunately,  
394 technical barriers related to the difficulty of quantification but also of identification and  
395 discrimination of the different isoforms by antibodies as all the Hsp70s described here have  
396 high sequence identities. Nevertheless, a western blot approach should allow a first  
397 quantitative assessment of Hsp70 constitutive protein levels. In order to determine whether  
398 there is a large native pool Hsp70 proteins in *E. superba*, which could explain the delayed  
399 Hsp70 responses described above Western blots were performed abdomen extracts from  
400 different species of eucarids from the Antarctic or Arctic regions. This was performed on the  
401 krill species *E. superba* (fished from different areas), *E. crystallorophias*, *Thysanoessa*  
402 *inermis* and the shrimp *Chorismus antarcticus* (Figure 7).

403 It is clear that the strongest labeling is present in the protein sample extracted from *E. superba*  
404 fished near Dumont d'Urville Station (lane 3). The other extracts are weakly stained or almost



405 absent, as shown by the results for the Antarctic shrimp (*Chorismus antarcticus*). These bands  
406 were globally at or slightly above the human HSPA control band estimated at 72 kDa. Due to  
407 their positions and according the amino-acid sequences deduced from transcriptomic data  
408 (Cascella et al. 2015; Clark et al. 2011), the bands shown in Figure 7 can be attributed to A  
409 isoform for the Antarctic species (EusHspA = 72.46 kDa, EucHspA = 72.56 kDa, as the B  
410 isoforms molecular weight are under 72kDa, 71.64 and 71.84 kDa respectively) and isoform  
411 B for *Thysanoessa inermis* (72.03 kDa), in which no A isoform was characterized  
412 (Huenerlage et al. 2016). The other isoforms do not appear to have been translated in  
413 sufficient quantity to allow visible labelling by the antibody. This observation, without  
414 constituting definitive proof, supports the hypothesis that *E. superba* maintains high  
415 constitutive levels of Hsp70 protein. The labeling is all the weaker as the species of krill  
416 considered have a significant early response in terms of expression of the mRNAs encoding  
417 these isoforms. In fact, *T. inermis* showed much faster and more Hsp70 intense responses to  
418 thermal shocks than Antarctic species, even though the corrected CT<sub>max</sub> values were very  
419 similar (Huenerlage et al. 2016). In the same way, *E. crystallorophias* appeared to be more  
420 reactive to temperature than Antarctic krill. The kinetics of response to thermal shock have  
421 not yet been carried out on *C. antarcticus*, however the CT<sub>max</sub> values were found to be much  
422 lower than those measured on *E. superba* and was particularly sensitive to the time factor,  
423 demonstrating a greater sensitivity to temperature variation (unpublished personal data).

424 The potential Hsp70A band could be preferentially involved in the response to cold shock in  
425 the natural environment (Peck et al. 2014). Interestingly, there is less native Hsp70A in *E.*  
426 *crystallorophias*, a species adapted to the colder environment of the Antarctic continental  
427 shelves. On the other hand, this protein is absent in the surveyed *T. inermis*, which lives in the  
428 warmer waters of the Arctico-boreal region. But most interesting is the labelling observed on  
429 the protein extract from *E. superba* specimens fished on the edge of the Antarctic Peninsula  
430 (Figure 7, lane 7). The water temperature in this region is regularly positive and it would have  
431 been reasonable to expect, on the basis of the stated hypothesis, that this Hsp70A band would  
432 have been less stained if the high quantity of Hsp70A in the sample in lanes 3 and 4 is due to  
433 cold acclimation. However, the difference is much more drastic, since the A band disappeared  
434 in favor of the potential band B (Figure 7, lane 7), similar to *T. inermis* (Figure 7, lane 6).

## 435 **General discussion and conclusion**

436 The high quantity of these Hsp70 isoforms in *Euphausia superba* constitutes a significant

437 advantage in response to either a heat or cold shock. The hypothesis that ectothermic animals  
438 living in cold environments favor the accumulation of Hsps to correct the damage directly  
439 linked to the problems of protein folding at low temperatures could be an explanation for this  
440 physiological behavior (Peck 2016; Place and Hofmann 2005). Thus, the delay in gene  
441 expression would find its origin in the presence of large constitutive amounts of Hsp proteins  
442 in Antarctic animals, in quantities that would be sufficient to manage the immediate damage  
443 caused by a moderate or an acute temperature shock. When the shock is prolonged and  
444 quantities and/or degradation of available Hsp70s become critical to correct cell damage  
445 caused by a thermal shock, the available mRNAs could be recruited via translation and at that  
446 moment new mRNAs would need to be transcribed. As a consequence, the higher the  
447 amounts of Hsp present as either protein or mRNA within the cell under normal conditions,  
448 the longer delay expected in the heat shock response (Bedulina et al. 2010). These  
449 assumptions correspond well with what was observed with acute heat shocks. Short shocks of  
450 1, 2, 3 or 6 hours did not cause significant up-regulation of Hsps whatever the intensity of the  
451 heat treatment. These results showed a general decrease in the mRNAs encoding the  
452 constitutive forms, probably involved in the synthesis of new Hsp70 proteins. However, there  
453 was significant up-regulation during post-shock recovery. This up-regulation of HSP70  
454 transcripts, which could, in the first instance, be attributed to recovery, were as likely to be  
455 due to the natural abundance levels of Hsp70s that have delayed the response, as  
456 demonstrated by the western blot results.

457 Nevertheless, the presence of large amounts of Hsp70, particularly in comparison with the  
458 benthic shrimp (*Chorismus antarcticus*), questions its justification as a universal adaptation of  
459 Southern Ocean endemic species. Reasoning that the large amount of Hsp70 protein in  
460 control animals would be linked to a potential protection against the effects of cold, Antarctic  
461 krill fished near the French base at Dumont d'Urville would suffer a permanent cold stress if  
462 an optimum temperature at  $0.5 \pm 1^\circ\text{C}$  is considered (Atkinson et al. 2006; Brown et al. 2010;  
463 Cuzin-Roudy et al. 2014; Mackey et al. 2012). Similarly, the labelling shown in *E. superba*  
464 from the Antarctic Peninsula could be attributed to a heat shock. Each of the animals studied  
465 would be outside its comfort zone and would therefore be subject to thermal stress. This result  
466 is a new evidence highlighting the role of HSPs in the adaptation of organisms to temperature  
467 other than through thermotolerance (Banerjee et al. 2014; Ulmasov et al. 1992). The species-  
468 specific differences in HSP70 isoforms may be more likely related to variations in thermal  
469 tolerance (Yamashita et al. 2004), and isoform expression may vary with regard to

470 thermotolerance (Hightower et al. 1999). In this context, the comparison of the two sister  
471 species, *E. crystallorophias* and *E. superba*, illustrates this duality in adaptive choices  
472 (Cascella et al. 2015; Papot et al. 2016). Indeed, there are differences in terms of  $CT_{max}$ ,  
473 response and antibody staining intensity of Hsp70s and therefore of the amount of proteins,  
474 but also in terms of selection pressure experienced by the two sister species *E. superba* and *E.*  
475 *crystallorophias* (Papot et al. 2016). These differences may be related to their geographic  
476 distribution, with ice krill preferring cold continental shelf waters with low temperature  
477 variation (Mackey et al. 2012), whereas *E. superba* lives in deep oceanic waters (Atkinson et  
478 al. 2008) and is more exposed to variable temperatures. *E. crystallorophias* would favor  
479 molecular thermotolerance to cold and thus a weak degradation of proteins in a cold  
480 environment and a limited involvement of Hsp70, whereas *E. superba*, due to a wider and  
481 more variable distribution, would be less cold adapted and would have developed a greater  
482 HSP response.

483 A similar case has been reported in amphipod species with different vertical distributions and  
484 thermotolerance capacities (Bedulina et al. 2013; Bedulina et al. 2010). The hypothesis put  
485 forward was that one of the two isoforms would only be synthesized under normal  
486 physiological conditions, whereas the second could be synthesized under normal conditions  
487 but also induced by heat stress. It seems similar in the case of krill, but the difference is more  
488 pronounced since although the two isoforms Hsp70A and B can be expressed together, they  
489 are mostly represented in the transcriptomes, they do not seem to be translated simultaneously.  
490 It was surprising, in view of the similar values of the FPKMs recorded for the two isoforms in  
491 the transcriptome of *E. superba* (Cascella et al. 2015), that the western blot revealed only one  
492 band in each of the samples of *E. superba* from east or west Antarctica. This should not be a  
493 recognition problem with the western blot since clearly both Hsp70 A and B isoforms can be  
494 recognized by the heterologous antibody. This difference could be attributed to the fact that  
495 the original *E. superba* transcriptome was made from a mixture of control and thermally  
496 stressed individuals. Nevertheless, the values obtained for qPCR for the controls are  
497 comparable for the two isoforms A and B. It could also be envisaged that the differences  
498 could be related to the genetic diversity of two populations. However, Antarctic krill is  
499 particularly genetically homogeneous despite its large population size and wide range,  
500 limiting differences potentially related to local conditions (Deagle et al. 2015). Based on  
501 these observations, differential translation as a function of environmental temperature would  
502 therefore remain a reasonable explanation for the results in this study. The experiment carried

503 out by western blot was conducted on control animals only, i.e. without the application of a  
504 heat shock. It will obviously be interesting to continue this study on animals having  
505 undergone heat stress in order to validate the hypothesis and to estimate the kinetics of  
506 appearance of the different isoforms. The *E. superba* sample (Figure 7, lane 4) was kept in an  
507 aquarium for 8 months at a temperature of 0.5°C before freezing, which corresponds to its  
508 theoretical thermal optimum (Kawaguchi et al. 2010). Although the Hsp70A staining seems  
509 less strong than for krill fished near Dumont d'Urville on the east coast, which is to be  
510 confirmed on a larger sample of individuals, it is nevertheless present and well-marked.

511 In summary, this differentiation of Hsp70 isoform protein expression between animals from  
512 West and East Antarctica raises more questions than it answers. However, it represents a new  
513 opportunity in the study of Hsp70s, which until now were not considered as strong markers of  
514 thermal stress in Antarctic krill species. Understanding the differential mechanisms of these  
515 thermoregulatory actors in *E. superba* is a prerequisite in the search for indicators of the  
516 biological impact of climate change on this global source of fisheries protein.

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613

## 614 Legends

615

616 Figure 1: A) Experimental protocol used during the thermal shock kinetics: 3 and 6 hours at  
617 3°C or 6°C (on Astrolabe board). B) Experimental protocol used for long term thermal shock  
618 at 3°C (AAD aquarium)

619 Figure 2: Curves representing the loss of mobility of a population of krill subjected to gradual  
620 temperature increase (0.1 °C / min, blue curve, n=43; 1°C / day, red curve, n=30; 1°C / 3days,  
621 green curve, n=30). CT<sub>max</sub> is the temperature at which 50% of mobile animals are reached

622 Figure 3: Mean normalized expression ratios of five *hsp70* isoforms in the muscle tissue of  
623 adult *E. superba* (n=6) during 3 and 6 hours continuous heat shock at 3°C or 6°C followed by  
624 6 hours of recovery at control temperature (0°C). Thermal shocks are presented on a white  
625 background, post shocks on a grey background. Values relate to the control group (specimens  
626 continuously kept at 0°C). Asterisk (\*): significant MNE difference to 0°C control  
627 temperature evaluated with a Kruskal–Wallis test. Figure was created using GraphPad Prism  
628 6.0

629 Figure 4: Mean normalized expression levels of five *hsp70* genes obtained by qPCR in *E.*  
630 *superba* during a heat shock of 3°C and 6°C whereas 0-hour group is control at 0°C. Heat  
631 shocks were carried out during 1 and 2 hours, and *hsp70* expressions measured at those times  
632 on n=6 individuals per group. *Hsp70* expressions were normalized by the 18S gene  
633 expression. To compare values, a Kruskal-Wallis test followed by a Dunn's test were used to  
634 compare control groups (0h) and shocked groups (1h and 2h). Significant differences between  
635 mean normalized expressions are indicated by asterisks (\*). Figure was created using  
636 GraphPad Prism 6.0

637 Figure 5: Mean normalized expression levels of five *hsp70* genes obtained by qPCR in *E.*  
638 *superba* during a heat shock of 9°C and 12°C whereas 0h group is control at 0°C. Heat shocks  
639 were carried out during 3h, and *hsp70* expressions measured at those time on n = 6  
640 individuals per group. *Hsp70* expressions were normalized by the 18S gene expression. To  
641 compare values, a Kruskal-Wallis test followed by a Dunn's test were used to compare  
642 control groups (0h) and shocked groups (9°C and 12°C). Significant differences between  
643 mean normalized expressions are indicated by asterisks (\*). Figure was created using  
644 GraphPad Prism 6.0

645 Figure 6: Mean normalized expression ratios of four *hsp70* isoforms in the muscle tissue of  
646 adult *E. superba* (n = 6) during continuous heat shock at 3°C for 6 weeks. Values relate to the  
647 control group (specimens continuously kept at 0°C). Asterisk (\*): significant MNE difference  
648 to 0°C control temperature evaluated with a Kruskal–Wallis test. Figure was created using  
649 GraphPad Prism 6.0

650 Figure 7: Western blot bands obtained using an anti-chicken monoclonal antibody for the  
651 detection of *Hsp70* proteins in abdomen muscle of *E. superba*. Species and origin are listed  
652 under the figure. At least 3 samples from each origin were tested.