HAL
archives-ouvertes.fr

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

Jean-Yves Toullec, Kévin Cascella, Stéphanie Ruault, Alexandre Geffroy, David Lorieux, Nicolas Montagne, Céline Ollivaux, Chi-Ying Lee

To cite this version:

Jean-Yves Toullec, Kévin Cascella, Stéphanie Ruault, Alexandre Geffroy, David Lorieux, et al.. Antarctic krill (Euphausia superba) in a warming ocean: thermotolerance and deciphering Hsp70 responses. Cell Stress and Chaperones, Springer Verlag, 2020, 25 (3), pp.519-531. 10.1007/s12192-020-01103-2 . hal-02862448

HAL Id: hal-02862448
https://hal.sorbonne-universite.fr/hal-02862448

Submitted on 9 Jun 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

Jean-Yves Toullec[1*], Kévin Cascella[1], Stéphanie Ruault[1], Alexandre Geffroy[1], David Lorieux[1], Nicolas Montagné[2], Céline Ollivaux[3], Chi-Ying Lee[4]

[1] Sorbonne Université, Faculté de Sciences, CNRS, UMR 7144, Adaptation et Diversité en Milieu Marin, AD2M, Station Biologique de Roscoff, 29682 Roscoff, France

[2] Sorbonne Université, Faculté de Sciences, institut of Ecology and Environmental Sciences Paris, iEES Paris, 75005 Paris, France

[3] Sorbonne Université, Faculté de Sciences, CNRS, UMR Laboratoire de Biologie Intégrative des Modèles Marins, LBI2M, Station Biologique de Roscoff, 29682 Roscoff, France

[4] Department of Biology, National Changhua University of Education, Changhua 50058, Taiwan

* Corresponding author: jean-yves.toullec@sb-roscoff.fr (JYT)

Keywords: Krill, *Euphausia superba*, heat shock, CT_{max}, *Hsp70* expression

Acknowledgements

KC received a PhD grant from the Emergence-UPMC 2011 research program and the "Région Bretagne". JYT benefited from funding provided by Institut Paul Emile Victor (IPEV) (KREVET program) and also from the "Région Bretagne" (SAD-1 - DRAKAR program). JYT would like to thank all the IPEV staff at the base Dumont d'Urville and Astrolabe's crew for their help during different Antarctic campaigns in Terre Adélie. JYT would also like to thank Dr. So Kawaguchi and Rob King for their invaluable advice and support in setting up the aquarium experiments at the AAD in Kingston, Tasmania. Many thanks to Drs. Juan Höfer (IDEAL, Chile) and Marcelo Gonzalez-Aravena (INACH, Chile)

30 for providing krill samples from western Antarctica. The authors would like to thank also the
31 reviewers for their constructive comments.

Abstract

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

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_{max} experiments**

158 CT_{max} 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_{max}
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_{max} 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_{max} 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_{max}
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 EF1α, 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 µl
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 µg protein well^{-1}). After running the electrophoresis (15 min at 15 mA gel^{-1}, 1 h
200 at 20 mA gel^{-1}), 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 µg protein well^{-1}) 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 peroxydase (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°C/min (Cascella et al. 2015). The interpretation of the CT_{max} curve of *E. superba* gave an
224 unexpectedly high value of 15.8 ±0.1°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 CT_{max} varied with the rate of
229 experimental warming, more precisely CT_{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°C
231 was significantly increased since the curves presented were obtained for intervals of 1 and 3
232 days (Figure 2).

233 Observed CT_{max} values were 14.7 ± 0.1°C and 13.9 ± 0.03°C for increments of 1°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 CT_{max} as a function of the time between each temperature increase
237 was surprising. Overall, the decrease in CT_{max} was approximately 1°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 antarticus*, a species phylogenetically closer than
241 most of the Antarctic invertebrates studied so far, with CT_{max} values of 11.6°C and 6.7 °C for
242 increments of 1°C every 10 minutes or every day respectively (personal and unpublished
243 data). In this example, the CT_{max} for a 1°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 *Hsp70*s 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_{max} 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_{max} 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 ± 1°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.

517

518 # References

519 Atkinson A et al. (2006) Natural growth rates in Antarctic krill (*Euphausia superba*): II.
520 Predictive models based on food, temperature, body length, sex, and maturity stage.
521 Limnology and Oceanography 51:973-987.
522 Atkinson A et al. (2004) Long-term decline in krill stock and increase in salps within the
523 Southern Ocean. Nature 432:100-103. doi:10.1038/Nature02950
524 Atkinson A et al. (2008) Oceanic circumpolar habitats of Antarctic krill. Marine Ecology-
525 Progress Series 362:1-23.
526 Banerjee D et al. (2014) Seasonal variation in expression pattern of genes under HSP70 :
527 Seasonal variation in expression pattern of genes under HSP70 family in heat- and
528 cold-adapted goats (*Capra hircus*). Cell Stress Chaperones 19:401-408.
529 doi:10.1007/s12192-013-0469-0
530 Baringou S et al. (2016) Diversity of cytosolic HSP70 Heat Shock Protein from decapods and
531 their phylogenetic placement within Arthropoda. Gene 591:97-107.
532 doi:10.1016/j.gene.2016.06.061
533 Bedulina DS et al. (2013) Expression patterns and organization of the hsp70 genes correlate
534 with thermotolerance in two congener endemic amphipod species (*Eulimnogammarus*
535 *cyaneus* and *E. verrucosus*) from Lake Baikal. Mol Ecol 22:1416-1430.
536 doi:10.1111/mec.12136
537 Bedulina DS et al. (2010) Sub-littoral and supra-littoral amphipods respond differently to
538 acute thermal stress. Comparative biochemistry and physiology Part B, Biochemistry
539 & molecular biology 155:413-418. doi:10.1016/j.cbpb.2010.01.004
540 Brown M et al. (2010) Temperature effects on the growth and maturation of Antarctic krill
541 (*Euphausia superba*). Deep-Sea Research Part Ii-Topical Studies in Oceanography
542 57:672-682. doi:Doi 10.1016/J.Dsr2.2009.10.016
543 Cascella K et al. (2015) Diversification, evolution and sub-functionalization of 70kDa heat-
544 shock proteins in two sister species of antarctic krill: differences in thermal habitats,
545 responses and implications under climate change. Plos One 10:1-23.
546 doi:10.1371/journal.pone.0121642
547 Clark MS et al. (2011) Antarctic krill 454 pyrosequencing reveals chaperone and stress
548 transcriptome. Plos One 6:e15919. doi:10.1371/journal.pone.0015919
549 Cuzin-Roudy J et al. (2014) Southern Ocean Euphausiids. In: De Broyer C. KP, Griffiths H.J.,
550 Raymond B., Udekem d'Acoz C. d', et al. (ed) Biogeographic Atlas of the Southern
551 Ocean. Scientific Committee on Antarctic Research, Cambridge, pp 309-320
552 Deagle BE et al. (2015) Antarctic krill population genomics: apparent panmixia, but genome
553 complexity and large population size muddy the water. Mol Ecol 24:4943-4959.
554 doi:10.1111/mec.13370
555 Denny M, Helmuth B (2009) Confronting the physiological bottleneck: A challenge from
556 ecomechanics. Integrative and comparative biology 49:197-201.
557 doi:10.1093/icb/icp070
558 Everson I (2000) Krill biology, ecology and fisheries. In. Blackwell Science, Oxford,
559 Feder ME, Hofmann GE (1999) Heat-shock proteins, molecular chaperones, and the stress
560 response: evolutionary and ecological physiology. Annu Rev Physiol 61:243-282.
561 doi:10.1146/annurev.physiol.61.1.243
562 Flores H et al. (2012) Impact of climate change on Antarctic krill. Marine Ecology-Progress
563 Series 458:1-19.
564 Hightower LE et al. (1999) Heat shock responses of closely related species of tropical and
565 desert fish. Am Zool 39:877-888.

566 Huenerlage K et al. (2016) Responses of the arcto-boreal krill species *Thysanoessa inermis* to
567 variations in water temperature: coupling Hsp70 isoform expressions with metabolism.
568 Cell Stress Chaperones 21:969-981. doi:10.1007/s12192-016-0720-6
569 Kawaguchi S et al. (2010) An experimental aquarium for observing the schooling behaviour
570 of Antarctic krill (*Euphausia superba*). Deep-Sea Research Part Ii-Topical Studies in
571 Oceanography 57:683-692. doi:Doi 10.1016/J.Dsr2.2009.10.017
572 Macaulay MC et al. (1984) Acoustic Characterization of Swarms of Antarctic Krill
573 (*Euphausia superba*) from Elephant Island and Bransfield Strait. Journal of
574 Crustacean Biology 4:16-44.
575 Mackey AP et al. (2012) Antarctic macrozooplankton of the southwest Atlantic sector and
576 Bellingshausen Sea: Baseline historical distributions (Discovery Investigations, 1928-
577 1935) related to temperature and food, with projections for subsequent ocean warming.
578 Deep-Sea Research Part Ii-Topical Studies in Oceanography 59:130-146.
579 Papot C et al. (2016) Divergent ecological histories of two sister Antarctic krill species led to
580 contrasted patterns of genetic diversity in their heat-shock protein (hsp70) arsenal.
581 Ecol Evol 6:1555-1575. doi:10.1002/ece3.1989
582 Peck LS (2016) A Cold Limit to Adaptation in the Sea. Trends Ecol Evol 31:13-26.
583 doi:10.1016/j.tree.2015.09.014
584 Peck LS et al. (2009) Animal temperature limits and ecological relevance: effects of size,
585 activity and rates of change. Funct Ecol 23:248-256. doi:10.1111/j.1365-
586 2435.2008.01537.x
587 Peck LS et al. (2014) Acclimation and thermal tolerance in Antarctic marine ectotherms. The
588 Journal of experimental biology 217:16-22. doi:10.1242/jeb.089946
589 Pfaffl MW et al. (2004) Determination of stable housekeeping genes, differentially regulated
590 target genes and sample integrity: BestKeeper - Excel-based tool using pair-wise
591 correlations. Biotechnol Lett 26:509-515.
592 Place SP, Hofmann GE (2005) Comparison of Hsc70 orthologs from polar and temperate
593 notothenioid fishes: differences in prevention of aggregation and refolding of
594 denatured proteins. Am J Physiol-Reg I 288:R1195-R1202.
595 Ravaux J et al. (2003) Heat-shock response and temperature resistance in the deep-sea vent
596 shrimp *Rimicaris exoculata*. J Exp Biol 206:2345-2354.
597 Ravaux J et al. (2012) Adaptation to thermally variable environments: capacity for
598 acclimation of thermal limit and heat shock response in the shrimp *Palaemonetes*
599 *varians*. J Comp Physiol B 182:899-907. doi:10.1007/s00360-012-0666-7
600 Schiermeier Q (2010) Ecologists fear Antarctic krill crisis. Nature 467:15. doi:467015a [pii]
601 10.1038/467015a
602 Schwenk K et al. (2009) Grand challenges in organismal biology. Integrative and comparative
603 biology 49:7-14. doi:10.1093/icb/icp034
604 Trivelpiece WZ et al. (2011) Variability in krill biomass links harvesting and climate
605 warming to penguin population changes in Antarctica. Proc Natl Acad Sci U S A
606 108:7625-7628. doi:10.1073/pnas.1016560108
607 Ulmasov KA et al. (1992) Heat-Shock Proteins and Thermoresistance in Lizards. P Natl Acad
608 Sci USA 89:1666-1670.
609 Yamashita M et al. (2004) Characterization of multiple members of the HSP70 family in
610 platyfish culture cells: Molecular evolution of stress protein HSP70 in vertebrates.
611 Gene 336:207-218.

612

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_{max} 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.