1 **Enjoying the warming Mediterranean: Transcriptomic responses to temperature**
2 **changes of a thermophilous keystone species in benthic communities.**

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23 Running title: Transcriptomic response to thermal changes

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27 ABSTRACT

28 Information about the genomic processes underlying responses to temperature changes is still limited
29 in non-model marine invertebrates. In this sense, transcriptomic analyses can help to identify genes
30 potentially related to thermal responses. We here investigated, via RNA-seq, whole-transcriptomic
31 responses to increased and decreased temperatures in a thermophilous keystone sea urchin, *Arbacia*
32 *lixula*, whose populations are increasing in the Mediterranean. This species is a key driver of benthic
33 communities' structure due to its grazing activity. We found a strong response to experimentally
34 induced cold temperature (7ºC), with 1,181 differentially expressed transcripts relative to the control
35 condition (13ºC), compared to only 179 in the warm (22ºC) treatment. A total of 84 (cold treatment)
36 and 3 (warm treatment) Gene Ontology terms were linked to the differentially expressed transcripts.
37 At 7ºC the expression of genes encoding different heat shock proteins (HSPs) was up-regulated,
38 together with apoptotic suppressor genes (e.g. *Bcl2)*, genes involved in the infection response and/or
39 pathogen-recognition (e.g. echinoidin) and ATP-associated genes, while protein biosynthesis and
40 DNA replication pathways were down-regulated. At 22 ºC neither HSPs induction nor activation of
41 the previously mentioned pathways were detected, with the exception of some apoptotic-related
42 activities that were up-regulated. Our results suggest a strong transcriptional response associated with
43 low temperatures, and support the idea of low water temperature being a major limitation for *A. lixula*
44 expansion across deep Mediterranean and northerner Atlantic waters.

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46 Keywords: Transcriptomics, RNA-seq, warming, benthic species, Mediterranean, thermal responses

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54 INTRODUCTION

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56 Predicting organismal responses to environmental shifts is one of the main priorities of
57 contemporary ecology (Calosi et al., 2017; King, McKeown, Smale, & Moore, 2018; Donelson et al.,
58 2019). During the last decades, scientific studies have linked global warming, characterised by both an
59 increase in mean temperatures and frequency of heat waves (Jordà, Marbà & Duarte 2012; Oliver et
60 al., 2018), to detrimental impacts on marine systems at different biological levels. At the ecosystem
61 level, impacts include the alteration of the whole ecosystem functioning as changes in food-wed
62 dynamics and in ecosystem productivity occur, together with biodiversity loss (see Smale et al., 2019;
63 Stillman, 2019). Mean temperature increases and heat waves also result in a number of lethal and sub-
64 lethal effects on particular species and populations, including coral reef bleaching, alteration of animal
65 migration routes and behaviour, and shifts of marine taxa distribution patterns, among many others
66 (e.g. Hoegh-Guldberg & Bruno, 2010; Deutsch, Ferrel, Seibel, Pörtner & Huey, 2015; Hughes et al.,
67 2017; King et al., 2018). At the individual level, when organisms are exposed to sub-lethal extreme
68 temperatures stress is likely to occur (e.g. Buckley & Huey, 2016). To compensate the negative
69 impacts related with stress, organisms develop different molecular and cellular mechanisms to
70 maintain physiological performance and cell homeostasis (Pörtner, 2002; Buckley & Huey, 2016).

71 The cellular and molecular pathways involved in thermal stress response in marine organisms
72 have been recently thoroughly studied. These studies showed changes in expression patterns of stress-
73 responsive genes, including gene pathways that regulate metabolism, oxidation-reduction processes,
74 cell cycle, and protein folding repair systems, among others (e.g. Gleason & Burton, 2015; Zhu et al.,
75 2016; Gierz, Forêt & Leggat, 2017; Kim, Kim, Choi & Rhee, 2017; Xu, Zhou & Sun, 2018; Zheng et
76 al., 2019). But, in marine invertebrates, most interest has focused on Heat Shock Proteins (HSPs)
77 (Feder & Hofmann, 1999; Tomanek, 2010; Kim et al., 2017). HSPs are chaperones highly conserved
78 during metazoan evolution that help proteins' folding and transport across cell membranes during non-
79 stressful conditions. They also refold and stabilise denatured proteins under different conditions of
80 stress (e.g. Matranga, Toia, Bonaventura & Müller, 2000; Di Natale et al., 2019). Nevertheless,

81 additional knowledge on the involvement of different genes and gene pathways, such as antioxidant
82 genes, apoptosis-associated and immune-associated genes, in ecologically relevant marine
83 invertebrates under thermal shifts is still desirable to unravel the whole mechanisms of thermal stress
84 response (Gleason & Burton, 2015; Zhu et al., 2016). One relatively recent approach to investigate
85 organismal rapid responses to environmental shifts, to identify potential physiological networks, and
86 to discover candidate genes involved in their responses, is to explore the whole transcriptional profiles
87 using RNA-seq techniques (e.g. Zhu, Zhang, Li, Que & Zhang, 2016; Evans, Pespeni, Hofmann,
88 Palumbi & Sanford, 2017; Xu et al., 2018). Although the relationship between mRNA transcript
89 abundance and protein abundance is still not clear (Feder & Walser, 2005), some studies have shown a
90 correlation between these two variables (Maier, Güell & Serrano, 2009). Changes in gene expression
91 are considered to be sensitive indicators of stress and potential predictors of organismal physiology
92 under experimental conditions (Feder & Walser, 2005; Buckley, Gracey & Somero 2006; Schoville,
93 Barreto, Moy, Wolff & Burton, 2012).
94 Among marine ecosystems, one the most impacted seas in the world is the Mediterranean
95 (Lejeusne, Chevaldonné, Pergent-Martini, Boudouresque & Pérez, 2010; Coll et al., 2010). This sea is
96 a hotspot of marine diversity subject to intense anthropogenic pressures (Claudet & Fraschetti, 2010;
97 Templado 2014), which interact with the ongoing global warming (Francour, Boudouresque,
98 Harmelin, Harmelin-Vivien & Quignard, 1994; Jordà et al. 2012). During the last three decades,
99 summer surface temperature (SST) has risen in the Mediterranean at a rate ranging from 0.25ºC per
100 decade in the western basin to 0.65ºC per decade in the eastern one (Marbà, Jordà, Agustí, Girard &
101 Duarte, 2015). High-resolution ocean models, considering a diversity of potential climate change
102 scenarios, have projected in all cases a significant increase in SST by the end of the century (see
103 Somot, Sevault & Déqué, 2006; 2008; Parry, Canziani, Palutikof, Van Der Linden, & Hanson, 2007;
104 Shaltout, & Omstedt, 2014). A Mediterranean seawater temperature rise represents a challenge for
105 most mediterranean taxa, reflected in sub-lethal effects linked to behavioural and physiological
106 responses (e.g. Anestis, Lazo, Pörtner & Michaelidis 2007; Prusina et al., 2014), lethal outcomes,
107 including mass mortality events associated to heat waves (e.g. Cerrano et al., 2000; Coma et al., 2009;

108 Garrabou et al., 2009), and the collapse of whole ecosystems along the warmest areas of the
109 Mediterranean (Rilov, 2016). This warming also brings about other indirect effects, accelerating the
110 entrance of warm-water alien species (Raitsos et al., 2010) and promoting the expansion of subtropical
111 species that naturally colonised the Mediterranean during different geological periods (Briand, 2008;
112 and examples of echinoderms in Wangensteen, Turon, Pérez-Portela & Palacín, 2012; Garcia-Cisneros
113 et al. 2017; Pérez-Portela et al., 2019).

114 The black sea urchin *Arbacia lixula* (Linnaeus 1758) has tropical affinities (Tortonese, 1965)
115 and an amphi-Atlantic distribution across shallow rocky ecosystems, being the Moroccan coast its
116 northern-most distribution limit in the east Atlantic. This sea urchin entered the Mediterranean basin
117 during the last Pleistocene interglacial period (Wangensteen et al., 2012; Pérez-Portela et al., 2019),
118 and it is now a common species across the whole Mediterranean (Tortonese, 1965; Palacín, Turon,
119 Ballesteros, Giribet & López, 1998). Densities of this species significantly increased in some
120 Mediterranean areas during the last decades (Francour et al., 1994, Harmelin et al., 1995; Hereu et al.,
121 2012), and it is among the key drivers structuring littoral communities due to its grazing activity
122 (Bonaviri, Fernández, Fanelli, Badalamenti & Gianguzza, 2011). The species is capable of shifting
123 littoral complex macroalgal beds into "barren grounds"- areas of high densities of sea urchins deprived
124 of erect seaweeds and dominated by crustose coralline algae - (Gianguzza et al., 2011; Bonaviri et al.,
125 2011). Several authors have predicted that the foreseen global warming might have a positive effect on
126 its reproduction output and larval survival (Francour et al., 1994; Gianguzza et al., 2014;
127 Wangensteen, Dupont, Casties, Turon & Palacín, 2013a; Wangensteen, Turon Caso & Palacín 2013b;
128 Visconti et al., 2017). This potential effect, if real, will represent a worrisome increase of the impact of
129 this sea urchin on littoral ecosystems in a near future (Gianguzza et al., 2011; Wangensteen et al.,
130 2013a, 2013b). On the other hand, it seems that the distribution of *A. lixula* is constrained by low
131 temperatures, like the low sea surface temperature provoked by the southward Portugal Current
132 (Martins, Hamann & Fiùza, 2002), which might be the cause of its absence along the south Atlantic
133 coast of Europe (Wangensteen et al., 2012). In this sense, experiments to investigate the potential of *A.*
134 *lixula* to invade deep seawaters, analysing the combined effect of pressure (from 1 atm to 250 atm)

135 and temperature (from 5ºC to 15ºC) on the survival of embryos and larvae, showed that the
136 combination of high temperatures and pressures, rather than temperature *per se*, might be the major
137 factor limiting the distribution in depth (Young, Tyler & Fenaux, 1997). It was then concluded that *A.*
138 *lixula* is more likely to invade deep habitats of the Mediterranean than open Atlantic ones, the latter
139 being characterised by lower deep temperatures (Young, Tyler & Fenaux, 1997). In contrast, more
140 recent studies have demonstrated higher mortality rates, larval growth abnormalities and significant
141 delays in settlement at the lowest experimental temperatures tested in this species (experimental
142 temperatures from 18ºC to 22ºC in Privitera, Noli, Falugi, & Chiantore, 2011; and from 16ºC to 19ºC
143 in Wangensteen et al., 2013a). According to these studies, the abundance of *A. lixula* in the
144 Mediterranean might be constrained by the low winter temperature of colder years, when mean
145 temperatures can drop to 11°C, because gonad maturation is then considerably impaired (Lejeusne et
146 al., 2010; Wangensteen et al., 2013a). But whereas the mentioned studies shed some light on the
147 effects of thermal variation on the early development stages of *A. lixula,* almost nothing is known
148 about its effects on the general performance of adult individuals, which can have different thermal
149 sensitivity (Buckley & Huey, 2016). The capability of adult individuals to acclimatise and endure
150 thermal changes is highly relevant from an evolutionary perspective. It not only affects their own
151 survival and/or fertility, but can also result in negative transgenerational carry-on effects on
152 hatchability and larval size of the next generation, which have been shown after prolonged periods of
153 parental exposure to elevated temperatures in some sea urchins (Zhao et al., 2018). In sea urchins,
154 transcriptomes from different tissue types and larval thermal stress responses have been characterised
155 (e.g. Runcie et al., 2012; Gillard, Garama & Brown, 2014; Gaitán-Espitia, Sánchez, Bruning &
156 Cárdenas, 2016; Pérez-Portela, Turon & Riesgo, 2016; Jia et al., 2017; Clark et al., 2019). But, to our
157 knowledge, transcriptome-wide screenings have never been used for measuring responses to thermal
158 variation in adult individuals of this animal group.

159 The aim of this study is to explore the short-term transcriptional response to thermal changes
160 in the subtropical sea urchin *A. lixula*. We set three specific objectives for our study: a) To quantify
161 and compare transcriptional responses to both high and low temperature treatments in *A. lixula* under

162 experimental conditions, b) To identify some of the most important candidate genes involved in rapid
163 thermal responses in sea urchins, and c) To determine the conservation of the genetic machinery
164 involved in thermal responses for both increase and decrease temperature challenges.
165 Many studies on global warming focus on the negative effect of rising temperatures, but in this study,
166 we worked under the hypothesis that *A. lixula* will experience higher stress when subjected to low
167 rather than to high temperatures. Based on previous transcriptional information from marine
168 invertebrates under thermal stress (e.g. Gleason & Burton, 2015; Zhu et al., 2016), we also expect
169 changes of expression patterns in different gene pathways during our temperature treatments,
170 including genes encoding HSPs, apoptosis and anti-apoptosis mechanisms, ATP-associated genes due
171 to an increase of energy demand to restore cell homeostasis, antioxidant genes since extreme
172 temperatures can increase cells' oxidative stress, and immune-associated genes (Xu et al., 2018). The
173 information obtained here will be relevant to understand the ecophysiological patterns of sea urchins
174 exposed to thermal challenges. We also discuss the significance of our findings for the foreseeable
175 ecological spread of this keystone species in the Mediterranean.
176
177 MATERIAL AND METHODS
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179 *Sea urchin collection*
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181 Adult specimens of *A. lixula* were collected by SCUBA diving in December 2012 from the shallow
182 subtidal population (5-8 m depth) of Punta Santa Anna, Blanes (41°40′22.47″N, 2°48′10.81″E, North-
183 western Mediterranean; Figure 1). Specimens were quickly transported to the laboratory (less than 2
184 Km away) in a cooler with seawater and oxygen tablets to keep stress induced by land transportation
185 to a minimum. Experiments were performed in the LEOV (Laboratory of Experimentation with Living
186 Organisms) facility of the Centre for Advanced Studies of Blanes (CEAB), equipped with an open
187 system of running seawater coming directly from a sea intake. Once in the laboratory, sea urchins
188 were measured with callipers and left to adjust for 48 hours in a common chamber with airflow and

189 flow-through running seawater at 13˚C, which was the sea temperature in Blanes at the collection
190 time. During these 48 hours animals had rocky surfaces available for grazing.
191
192 *Experimental design*
193
194 To quantify rapid transcriptomic responses of *A. lixula* under thermal assays, we exposed adult
195 sea urchins (test diameter 40 to 50 mm) to three different treatments under controlled conditions in
196 laboratory for 20 hours: control (CT) with sea water at 13˚C± 1˚C, sea water temperature at 7˚C±
197 0.5˚C (T7), and sea water temperature at 22˚C ± 0.5˚C (T22). We set the temperature exposure time to
198 20 hours because previous experiments of thermal stress responses in other marine invertebrates
199 demonstrated maximum peaks of expression between the first 6- 24 hours, depending on the genes
200 (e.g. Zhu et al., 2016; Kim et al., 2017).
201 It is important to note that our goal was to submit the test organisms to an acute thermal
202 change to measure their responses, not to mimic highest or lowest seasonal temperatures in the area.
203 The treatment temperatures were chosen to represent an important shift with respect to the controls
204 (13ºC, the surface water temperature at this location when sea urchins were collected in wintertime)
205 while remaining within realistic values for our area of study, the NW Mediterranean. Thermal
206 sensitivity and resistance of organisms are not constant over time and often shift in response to
207 seasonal conditions (Buckley & Huey, 2016). The temperatures chosen, therefore, would have been
208 different had we performed the trials at other seasons as they were contingent on current conditions at
209 the time of the experiment. The average summer surface temperatures in the Mediterranean range
210 from 22ºC to 28ºC, with the lowest values at the north Aegean, Alboran Sea, and NW Mediterranean
211 (Pastor 2012; Marbà et al., 2015). The global average for the coldest month of the year (February) in
212 the Mediterranean is 14.5ºC, with a lower average value (12º-13ºC) found at the NW Mediterranean
213 (Pastor 2012) (see Supplementary Information S1). Since the species' thermal history can determine
214 the thresholds of stress response (Osovitz & Hofmann 2005) and thermal sensitivity can change over
215 the seasons, we made a preliminary assessment of the tolerance limits of our NW Mediterranean

216 population at that time of the year (so-called here “trials”), with several temperatures assayed over a
217 20 hours period and visual inspection of the state and activity level of 10 sea urchins per temperature
218 treatment. Specimens used for the trials were not used for further experiments and were returned to the
219 sea after experimentation, nor were samples collected for transcriptomic analysis during the trials. For
220 the trials, we used 22ºC, 24ºC, and 26ºC as upper thermal limits, and 12ºC, 9ºC and 7ºC as lower
221 limits. 7˚C and 22˚C marked the lower and upper thresholds, respectively, at which all individuals
222 used for the trials remained alive, visually healthy (intact skin, no algae or microorganism colonies
223 growing up over the animal surface and no massive spine lost) and active (feet and spines movement).
224 For the cold treatment, 7ºC (a decrease of 6ºC relative to the control) was the limit temperature
225 achievable in winter in shallow embayments in the NW Mediterranean (e.g. Ordoñez et al. 2015),
226 while for the warm treatment we increased temperature by 9ºC (relative to the control), being 22º-23ºC
227 the conditions encountered in mid-summer in the study area (e.g. Pastor 2012; Marbà et al., 2015; De
228 Caralt, González, Turon & Uriz, 2018). Over 22ºC, experimental animals either died or presented
229 clear signs of infection with microorganism colonies over the skin and/or massive loss of spines. We
230 emphasize that, while sea urchins thrive at this temperature and higher in summer, we were
231 performing an acute exposure treatment during wintertime, so we had to adjust our treatments
232 accordingly.

233 Our experimental design for transcriptomic analysis consisted of two different experiments: A
234 “Low temperature” experiment comparing the control condition at 13˚C ± 1˚C and experimental
235 condition at 7˚C ± 0.5˚C, hereafter named as “Control vs T7”, and a “High temperature” experiment
236 comparing the control condition at 13˚C ± 1˚C and experimental condition at 22˚C ± 0.5˚C, hereafter
237 named as “Control vs T22” (see Figure 1). Samples used as control condition were the same for both
238 experiments, since all treatments were run at the same time and laboratory. After the acclimation
239 period of 48 hours, each sea urchin was placed in an independent aquarium to avoid interactions
240 among specimens. Each aquarium had constant airflow and the seawater temperature was set at the
241 required temperature (13ºC, 7ºC or 22ºC) prior to adding the sea urchins. Temperature of the aquaria
242 was controlled with HOBO loggers (one per aquarium). Aquaria with different treatments were

243 randomly allocated across the wet-lab space to avoid any bias related to their spatial distribution.
244 Animals were not fed during the 20 h of the experimental time, and seawater pH (8.1) was monitored
245 during the experiments. Eight different replicates (specimens) per treatment were included, although
246 for gene expression analyses only six of them were processed. The sample size of 8 was used to ensure
247 an even proportion of sexes in the specimens analysed (since sex determination can be only performed
248 *a posteriori* after dissection), and indeed we processed for transcriptomic analyses 3 males and 3
249 females per treatment. After the 20 hours of treatment, sea urchins were removed from the aquaria,
250 quickly dissected under RNAase free conditions, and coelomocyte fluid collected and processed as
251 explained in the next section.
252 For sex determination we used histological techniques. One gonad per individual was obtained
253 and preserved in 4% formaldehyde. Gonad samples were washed in distilled water, dehydrated,
254 embedded in paraffin, cut in 5 μm sections using a Microm HM325 Microtome, and stained in
255 haematoxylin–eosin as described in Wangensteen et al. (2013b) and Garcia-Cisneros et al. (2017). Sex
256 was then determined under the optical microscope.
257
258 *Coelomocytes collection and RNA sequencing*
259
260 Coelomocytes consist of several cell types contained in the coelomic fluid and are immune effectors in
261 echinoderms (Matranga et al., 2000; Smith et al., 2018). They have been used as biomarkers of stress
262 due to their prompt response to changing environmental conditions (Matranga et al., 2000, Matranga,
263 Bonaventura & Di Bella, 2002; Matranga et al., 2005; Pinsino et al., 2008) that can reduce the
264 protective capacity of these cells and rapidly induce activation of the heat shock proteins expression
265 (Matranga et al., 2000; Pinsino et al., 2008). Additionally, these cells showed higher thermal response
266 capacity than other tissues in sea urchins (e.g., digestive tissues, Gonzalez et al., 2016), and protocols
267 for extraction of high quality RNA and high throughput sequencing have been developed for this
268 tissue type in *A. lixula* (Pérez-Portela & Riesgo 2013; Pérez-Portela et al., 2016).
269 Five millilitres of the coelomic fluid of each specimen (a total of 18 specimens; six per

270 treatment) was collected using a sterile syringe inserted through the peristomic membrane, taking care
271 not to puncture the gut. The fluid was then centrifuged, and all fresh cellular components
272 (coelomocytes) gathered and quickly embedded in TRizol reagent (Invitrogen, www.invitrogen.com).
273 Total RNA was directly extracted from coelomocytes following a protocol previously optimized for
274 this species (Pérez-Portela & Riesgo 2013 and Pérez-Portela et al., 2016). Integrity of total RNA and
275 potential DNA contaminations were initially evaluated by visualizing the 28S rRNA and 18S rRNA
276 bands into a 1% agarose gel in 1x TAE Buffer. Concentration of the RNA extracts was assessed in a
277 Hellma spectrophotometer (Hellma Analytics), and total RNA extracts were also run in an Agilent
278 2100 Bioanalyzer (Agilent Technologies) at the Scientific and Technical Services of the University of
279 Barcelona for quality measurements. High quality RNA (RINs over 8.5) samples were sent to the
280 National Centre of Genomic Analyses of Barcelona (CNAG) for mRNA isolation, cDNA library
281 construction, normalization and sequencing.
282 Isolation of mRNA and cDNA library preparation for each of the 18 specimens were
283 performed using the Illumina TruSeq RNA Sample Prep Kit (Illumina, Inc.) following the
284 manufacturer's recommendations, with an input of 800-900 ng of mRNA, and average insert size of
285 the libraries of 300 bp. Quality and concentration of the 18 cDNA libraries was controlled with
286 Ribogreen Assays in a NanoDrop 3300™ Fluorospectrometer (Thermo Fisher Scientific,
287 www.thermofisher.com). The 18 libraries (6 per treatment) were multiplexed with Illumina barcodes,
288 5 libraries per lane were sequenced on an Illumina HiSeq2000 Sequencer, and 101 base paired-end
289 reads were generated. The 18 libraries from different treatments were randomised across Illumina
290 lanes.
291
292 *Sequence processing and* de novo *assembly*
293
294 The software FASTQC v. 0.10.0 (www.bioinformatics.babraham.ac.uk) was used to visualise and
295 measure the quality of the raw reads generated in the HiSeq2000. Adapters and bases with low quality
296 (phred scores <33) were trimmed off, and a length filter was applied to keep only sequences of >25

297 bases using TrimGalore v. 0.2.6 (www.bioinformatics.babraham.ac.uk). High-quality reads were re-
298 screened in FASTQC to ensure a good quality of the samples after trimming. A basic scheme of the
299 most important steps of our pipeline is presented in Figure 2.

300 Two *de novo* assemblies, hereafter named as "CT+T7" and "CT+T22", one per experiment
301 ("Control *vs* T7" and "Control *vs* T22", respectively), were separately built up as reference for gene
302 expression analyses. Due to technical difficulties and the low quality of two libraries, for gene
303 expression only 5 samples could be used for each of the T7 and T22 treatments (see details in Results
304 section and Figure 1). Nevertheless, these two libraries discarded for gene expression could be used
305 for the assembly of the respective references. The assemblies were performed separately for each
306 experiment to ensure that the corresponding reference is the most comprehensive for each particular
307 experiment. The *de novo* assemblies were performed with the software Trinity (Grabherr et al., 2011),
308 which allows detecting differentially spliced isoforms, with default parameters for this software. Only
309 contigs with a minimum length for reported transcripts of 200 bp and at least 10x coverage were
310 retained for the assemblies.

311 The two *de novo* assemblies were separately blasted against a selection of the *nr* database of
312 NCBI containing only proteins from Metazoa (blastx) using BLAST (Altschul et al., 1997) with a cut-
313 off E value of $1e^{-5}$. The highest scoring blast hit was used to assign a gene name to each contig. *De*
314 *novo* assemblies were also blasted against both a database containing proteins of bacteria (blastx), and
315 a database of ribosomal DNA of bacteria (blastn) obtained from NCBI to remove bacterial
316 contaminations. Sequences with blast hit exclusively against proteins and nucleotides of bacteria were
317 eliminated from the datasets.

318 Blast results against Metazoa served as a database for annotation of transcripts differentially
319 expressed between treatments (see below). Moreover, Blast results of the assemblies were used to
320 retrieve Gene Ontology (GO) terms with BLAST2GO (Conesa et al., 2005) under different categories:
321 biological processes, molecular function and cellular component, which are hierarchically organized
322 into different levels (see Figure 2). The completeness of the reference transcriptomes was assessed

323 with BUSCO (Benchmarking Universal Single-Copy Ortholog) against the eukaryotic and metazoan
324 databases (Simão, Waterhouse, Ioannidis, Kriventseva & Zdobnov, 2015).
325
326 *Differential expression analyses and annotation*
327
328 Reads from all replicates in each experiment were aligned against the corresponding "reference"
329 transcriptome as per experiment (see Figure 2). Paired reads after trimming were mapped using
330 Bowtie2 v. 2.2.1 (Langmead & Salzberg, 2012) as implemented in Trinity (Grabherr et al., 2011).
331 RSEM v. 1.2.11 (Li & Dewey, 2011) was then run to generate a table with read counts, and unmapped
332 reads were discarded. In the "reference" transcriptomes, transcripts of the same trinity component
333 were treated as different isoforms. We retained information of differential expression of all isoforms
334 detected for a given gene (or component) because they may have different functions.
335 Differential expression (DE) analyses of the two experiments were performed with the
336 package DESeq2 (Love, Huber & Anders, 2014) in R v 3.2.1 (R Development Core Team 2008).
337 Before performing the analyses, preliminary tests to investigate differences in gene expression
338 between sexes and treatments were performed. No significant differences in response to treatments
339 were observed between males and females, and "sex" was not considered as a variable in further
340 analyses.
341 Before analysing differential gene expression, read counts were normalized, and then a
342 negative binomial model was fit to accurately estimate differential expression. The significance value
343 for multiple comparisons was adjusted to 0.01 with the function "padj" (Benjamini-Hochberg
344 adjustment) as implemented in DESeq2. Transcripts with significantly different expression values
345 relative to the controls will be hereafter called "DE" transcripts. Component Analyses (PCAs) were
346 performed and plotted with the same package to visualize variation of expression levels among
347 samples and treatments. Visualization of the significant outcomes of isoforms differentially expressed
348 (up- and down-regulated) between treatments of each experiment was obtained with a heatmap
349 performed with the "gplots" package of R (Warnes, Bolker, Bonebakker & Gentleman, 2016).

350 Using the GO annotation results from the *de novo* assemblies of the two experiments, we
351 obtained the GO terms associated to the differentially expressed isoforms, which were then input
352 (together with their associated log2foldchange) to the REVIGO web server (Supek, Bošnjak, Škunca,
353 & Šmuc, 2011) to obtain summaries of GO terms. Results were graphically represented with the
354 "treemap" R package. Size of the rectangles was adjusted to reflect the log2foldchange in REVIGO.
355 Differentially expressed isoforms without blast hit, unknown function and/or without annotation for
356 each experiment were assessed with the InterProScan 5 software (Jones et al., 2014), which predicts
357 protein family membership and the presence of functional domains and sites, at the Superfamily level
358 (De Lima Morais et al., 2011). The InterproScan was run as implemented in the Blast2GO software
359 with default parameters. We finally merged the results of the associated GO terms and those from
360 InterProScan with the purpose of increasing our knowledge of coelomocyte gene functions and GO
361 annotations.

362 In order to identify common genes and/or isoforms differentially expressed under temperature
363 increase and decrease, the *de novo* assemblies of both experiments, that assigned different transcript
364 names to all isoforms, were blasted against each other using BLASTn.

365

366 RESULTS

367

368 *Data filtering and* de novo *assembly*

369

370 A total of 18 RNA-seq datasets were used for *de novo* assembling (see Figure 2), and 16 datasets for
371 quantifying transcriptomic responses in *A. lixula* (see Figure 1) since one sample from experiment T7
372 and another from T22 were discarded for gene expression analyses because of their low quality.
373 Datasets have been deposited in Mendeley Data (doi.org/10.17632/5673n552yj.1). The number of
374 trimmed reads used for *de novo* assembly, as per sample replicate and treatment, are detailed in Tables
375 1 and 2. All replicates had over 26 million reads.

376 The *de novo* assembly "CT+T7", used as a reference for the "Control *vs* T7" experiment,

377 included 141.5 Megabases that rendered 211,650 transcripts (including both genes and their different
378 isoforms), and 19.6% of them had blast hit with known proteins of metazoans (see species blast hit
379 distribution in Supplementary Information S2). The reference assembly "CT+T22" for the "Control *vs*
380 T22" experiment included 147.4 Megabases, and rendered 219,655 different transcripts, from which
381 17.9% had blast hit (see species blast hit distribution in Supplementary Information S2). Both *de novo*
382 assemblies were very comparable (and had 99,5% transcripts in common), presenting relatively high
383 N50 values, between 1,102 and 1,114, meaning that over 50% of the transcripts were longer than
384 1,100 bases. Details of the *de novo* assemblies for the two different experiments are presented in Table
385 1. Both, "CT+T7" and "CT+T22", showed high completeness when compared with BUSCO
386 conserved ortholog databases of eukaryotes and metazoans (see Table 3). For the reference
387 assemblies, "CT+T7" and "CT+T22", 194 and 4,293 transcripts, respectively, had blast hits against
388 proteins and/ or nucleotides of bacteria and were removed from subsequent analyses. In fact, most
389 differences between the reference assemblies "CT+T7" and "CT+T22" were due to the amount of
390 bacterial transcripts.

391

392 *General results of differential expression analyses*

393

394 The differential expression analyses revealed changes in gene expression between controls and
395 temperature treatments in both experiments, "Control *vs* T7" and "Control *vs* T22", but with a
396 remarkable difference in the magnitude of the transcriptomic responses, which was over 6 times
397 higher in number of differentially expressed (DE) transcripts in the former experiment, as explained
398 below. We also detected differences in gene expression among different isoforms of the same genes.

399

400 In the "Control *vs* T7" experiment, we detected 1,181 DE transcripts between CT and T7,
401 being 720 transcripts up-regulated at T7 (61% of the total DE transcripts) and 461 transcripts down-
402 regulated at T7 (49% of the total DE transcripts) (see Figure 3). 445 transcripts (37.7 % of the total DE
403 transcripts) had blast hit and known function (see Table 4), including 28 transcripts within the top 50

404 most significant DE (see Supplementary Information S3). Regarding different isoforms, over all DE
405 transcripts (potential genes), 176 presented different isoforms (see Supplementary Information S3).
406 Ten genes presented all isoforms DE between CT and T7, whereas the other 166 genes only showed
407 some of their isoforms differentially expressed between treatments.

408 The number of DE transcripts in the "Control *vs* T22" experiment was much lower than in the
409 "Control *vs* T7" experiment, with only 179 DE transcripts, being 57 transcripts up-regulated (32% of
410 the total DE transcripts) and 122 transcripts down-regulated (68% of the total DE transcripts) in the
411 T22 treatment (see Figure 2). Only 35 transcripts (19.7 % of the total DE transcripts) had annotation
412 and known function (Table 4), 10 of them within the top 50 most significant DE. Of these 35
413 transcripts, 27 had different isoforms, and in all cases only one of their isoforms was DE between CT
414 and T22 (see Supplementary Information S3). A complete list of differentially expressed, annotated
415 transcripts for both experiments is presented as Supplementary Information (S3), including transcript
416 identification code (id), logarithm of the fold change, adjusted *p*-value with FDR correction obtained
417 from the expression analyses, gene description, number of isoforms found and transcripts with known
418 function within the top 50 most significant DE (* Top 50 DE). Fourteen DE transcripts were common
419 between experiments (see Figure 3) and most of them featured opposite responses between treatments.
420 Only four of these transcripts had annotations (*fam-55cc*, *tripartite motif-containing protein* 3, and *wsc*
421 *domain- containing protein 1* with opposite responses in T7 and T22, whereas the *histone-lysine n-*
422 *methyltransferase prdm 9* was down-regulated in both temperature treatments of the two experiments,
423 T7 and T22).

424 Figure 4 represents the hierarchical clustering of all transcripts related to their expression
425 differences between treatments for each experiment (heatmaps), and Figure 5 the corresponding
426 PCAs. The heatmaps and PCAs showed, in general, little differentiation between replicates of the
427 same treatment, and large differences in transcript expression between treatments. Only one of the
428 control replicates had a mixed pattern of expression between that of the other control samples (Control
429 replicate 1, see Figure 4) and those from treatment 22ºC, and clustered together to the T22 samples on
430 the PCA (see Figure 5). However, this control sample did not follow the same trend in the other

431 experiment, “Control *vs* T7”.

432 A total of 84 and three GO terms were found associated to the differentially expressed 433 transcripts in the “Control *vs* T7” and “Control *vs* T22” experiments, respectively (Table 4). The 434 InterProScan could only predict information of protein domains in six uncharacterised transcripts of 435 the “Control *vs* T22” experiment. In Figures 6 and 7 the up- and down-regulated GO categories 436 associated to DE transcripts from the two experiments are depicted. These GO terms were not equally 437 represented among categories between up- and down-regulated DE transcripts, or between 438 experiments. For the experiment “Control *vs* T7” the most important up-regulated GO term categories 439 for Biological Process (BP) were “tyrosine metabolism” (including “positive regulation of apoptotic 440 process”), “peptidyl-tyrosine dephosphorylation”, “protein folding” and “ATP hydrolysis coupled 441 proton transport”; “proton-transporting V-type ATPase-V0 domain” and “sarcoplasmic reticulum” for 442 Cellular Component (CC), and “GTP binding”, “protein tyrosine phosphatase activity”, “Protein 443 tyrosine phosphatase activity”, “sulfo-transferase activity”, “hydrogen ion transmembrane transporter 444 activity” and “lipid binding” (among others) for Molecular Function (MF) (Figure 6). The most 445 important down-regulated GO categories for BP were “neurotransmitter transport”, “Intracellular 446 signal transduction” and “protein O-linked glycosylation”; “nuclear origin of replication recognition 447 complex”, “cell”, “intracellular” and “integral component of membrane” for CC, and “protein-N-448 acetylglucosaminyltransferase activity”, “sequence- specific DNA binding”, NAD-dependent histone 449 deacetylase activity” and “zinc ion binding” (among others) for MF (Figure 6). For the experiment 450 “Control *vs* T22” only GO information for down regulated transcripts could be obtained and, among 451 them, the most important DE categories were “notch signalling pathway”, “multicellular organismal 452 development” for BP, “integral component of membrane”, “membrane”, and “SAGA-type complex” 453 for CC, and “calcium ion” and “protein binding” for MF.

454

455 *Differentially expressed genes involved in thermal stress, apoptotic processes and immune responses* 456 *in* Arbacia lixula

457

458 At 7˚C, the production of different heat shock proteins was up-regulated, including different
459 transcripts for the Heat Shock family proteins: an inducible *Hsp70*, and *Hsp71*, *Hsp90* and the *Dnaj*
460 *homolog subfamily c member 21(DNAJC21)*, which encodes a member of the DNAJ heat shock
461 protein 40 family (*Hsp40*) (see Supplementary Information S3 for *Hsp40* transcripts and foldchanges:
462 c256938_g1_i3, log2foldchange= 2.98; c260821_g2_i1, log2foldchange= 1.35; c260821_g1_i2,
463 log2foldchange= 3.05; c264479_g1_i1, log2foldchange= 7.35; c249691_g1_i1, log2foldchange= 1.91;
464 c271252_g1_i1, log2foldchange= 3.69) acting as a co-chaperone of *Hsp70* (Supplementary
465 Information S3). In addition, the receptor of stress *Wsc domain-containing protein 1* was found down-
466 regulated at 7˚C and up-regulated at 22˚C (Supplementary Information S3: c266025_g2_i1,
467 log2foldchange= -1.19; and c265343_g1_i1, log2foldchange= 1.84, respectively).

468 Several genes from the apoptotic gene complements were differentially expressed between
469 controls and T7. They included the *Bcl2* (up-regulated in T7, Supplementary Information S3:
470 c263429_g1_i1, log2foldchange= 2.17; and c271119_g2_i1, log2foldchange= 1.73), *sequestosome 1*
471 (up-regulated in T7, Supplementary Information S3: c257995_g1_i1, log2foldchange= 3.72) and *fas-*
472 *associating death domain-containing protein* and *death ligand signal enhancer* (down- and up-
473 regulated in T7, respectively; Supplementary Information S3: c268119_g1_i3, log2foldchange= -1.48
474 and c270362_g1_i1, log2foldchange= 1.99). In T22, we found upregulation of *immediate early*
475 *response 3-interacting protein 1-like* (Supplementary Information S3: c276658_g1_i2,
476 log2foldchange= 1.55).

477 At 7˚C, there was an up-regulation of genes involved in the innate immune response identified
478 as *echinoidin*, *senescence associated-gene* and *Tripartite motif-containing protein 3* (TRIM)
479 (Supplementary Information S3: c258741_g1_i1, log2foldchange= 5.35; c150071_g1_i1,
480 log2foldchange= 6.02; c273778_g2_i1, log2foldchange= 2.90). In addition, the genes *interleukin-17*
481 and cytohesin-like were also upregulated in T7 (Supplementary Information S3: c239836_g1_i1,
482 log2foldchange=6.22; and c263807_g1_i1, log2foldchange=1.77, respectively).

483

484 DISCUSSION

485

486 The response of marine organisms to thermal shifts is likely different across the species' range of
487 distribution (Donelson et al. 2019). In our study, we investigated transcriptional responses of a
488 keystone species, the black sea urchin, in the northern part of its range of distribution (NW
489 Mediterranean). We found contrasting responses to low (7˚C) and high (22˚C) temperatures, with the
490 former eliciting a much stronger reaction. Such differences were related to both the magnitude of the
491 transcriptional response (e.g. number of up- and down- regulated transcripts and gene expression fold-
492 change) and the diversity of genes and pathways involved in these responses.

493 The capacity of ectotherm species to thrive across wide temperature ranges is, in part, based
494 on their ability to modulate the expression of genes encoding proteins involved in the physiological,
495 metabolic and cellular stress responses (Stillman, 2003; Runcie et al., 2012; Tomanek, 2010; Kim et
496 al., 2017). Resistance to acute sublethal temperatures is an adaptive trait that varies among species of
497 the same genus from different latitudes and habitats (Stillman, 2003; Yao & Somero, 2012). In
498 general, marine tropical species are more heat tolerant than their temperate and cold counterparts
499 (Somero, 2010). Paradoxically, analyses of both marine and terrestrial ectotherms suggest that
500 tropical, or the warmest-adapted species, may be more threatened by global warming because they live
501 closer to their upper physiological thermal limit, and have higher metabolic rates that accelerate
502 quicker than in colder species under rising thermal conditions (e.g. Stillman, 2003; Somero, 2010).
503 According to this expectation, *A. lixula*, a heat tolerant species with sub-tropical affinities (Tortonese,
504 1965; Wangensteen et al., 2012), could be threatened by global warming across the warmest areas of
505 its geographical distribution (Elmasry et al., 2015; Rilov 2015), where it might be closer to its thermal
506 physiological limits. However, in the Northwestern Mediterranean this species is in the coldest part of
507 their range of distribution, which encompasses both sides of the tropical and subtropical Atlantic
508 (Wangensteen et al. 2012), and thus it could be more limited by cold temperatures. Current
509 Mediterranean sea warming may be removing thermal limitations for this species (Francour et al.,
510 1994; Gianguzza et al., 2014; Wangensteen et al., 2013a, 2013b; Visconti et al., 2017) allowing an
511 increase in its abundance in the Mediterranean.

512 In general, it is difficult to determine whether changes of expression in particular genes have
513 important functional consequences, because for each gene the threshold for metabolic and
514 physiological downstream effects can be different, and relatively small changes in gene expression of
515 only a few genes can be as functionally important as larger changes in other genes (Oleksiak, Roach
516 & Crawford, 2005). However, the overall changes of gene expression patterns found in *A. lixula*, the
517 number of genes differentially expressed, and the clustering of one control individual with the 22˚C
518 experimental individuals at the PCA and heatmap, indicates a lower transcriptional response to rapid
519 temperature increases in this subtropical species.

520 Decreasing temperatures elicited the activation of genes related to metabolism changes, pro-
521 and anti-apoptotic mechanisms, and immune responses in coelomocytes of *A. lixula*. Among the
522 upregulated genes related to the stress response at 7˚C, we detected the *Hsp71*, *Hsp90*, an inducible
523 *Hsp70*, and *Hsp40*; being the last one a co-chaperone of the *Hsp70*. The protein *Hsp40s* stimulate the
524 ATPase activity of *Hsp70s* and targets unfolded proteins to *Hsp70s* (Ngosuwan, Wang, Fung &
525 Chirico, 2003). In general, these HSP chaperones are involved in the strong and mild thermal stress
526 response and protein folding reaction to avoid protein denaturation in sea urchins, either in adult or
527 early development stages and eggs (e.g. Matranga et al., 2000, 2002; Runcie et al., 2012; González,
528 Gaitán-Espitia, Font, Cárdenas & González-Aravena, 2016). Their presence might be involved in the
529 wide thermal distribution of some particular marine species (see Zhu et al., 2016, and references
530 herein), and the HSP family seems to be a mechanism to cope with the stress associated with cold, and
531 with temperatures existing along the lower-end of thermal tolerances for *A. lixula* (e.g. NW
532 Mediterranean). On the other hand, no overexpression of genes encoding HSPs was detected at 22˚C
533 in *A. lixula*.

534 Under conditions of thermal stress, protein refolding by HSPs may not be efficient enough,
535 and misfolded protein degradation can be necessary to restore cell homeostasis (Mosser et al., 2000).
536 Therefore, other mechanisms such as proteolysis to eliminate disfunctional proteins via the *Ubiquitin*
537 proteosome pathway, and finally apoptosis to eliminate damaged cells, can be activated (Somero,
538 2010; Logan & Somero, 2011; Zhu et al., 2016). We only detected signs of *Ubiquitin* proteosome

539 pathway activation in the 7˚C treatment, with the up-regulation of the gene *sequestosome 1*
540 (Supplementary Information S3), which is an autophagosome cargo that detects proteins for autophagy
541 previously identified in echinoderms (Bitto et al. 2014), and the *e3 ubiquitin-protein ligase*, which
542 targets damaged proteins for transport and degradation by the proteasome (Ardley & Robinson, 2005).
543 In addition, we observed differential expression of several apoptosis-associated genes in both
544 treatments, 7˚C and 22˚C. Several studies demonstrated that sea urchins hold a complex apoptotic
545 system (Agnello & Roccheri 2010; Lesser, 2012). We found transcriptional changes at 7˚C in
546 apoptosis suppressor genes such as the *Bcl2* (up-regulated, Supplementary Information S3), widely
547 distributed in different marine invertebrates (see Lesser, 2012), and in genes containing death domains
548 (down-regulated: *fas-associating death domain-containing protein* and *death ligand signal enhancer,*
549 Supplementary Information S3) that induce cell apoptosis through the regulation of caspase activation
550 (Agnello & Roccheri 2010; Zhu et al., 2016). These findings suggest the activation of some particular
551 pathways to control the programmed cell death at low temperatures. The up-regulation at 22˚C of the
552 gene *immediate early response 3-interacting protein 1-like* (Supplementary Information S3), which is
553 a molecule involved in protein transport between the Sarcoplasmic reticulum and Golgi apparatus and
554 that mediates apoptosis in human cells (https://www.uniprot.org), suggests that apoptosis is also
555 occurring as a response of increased experimental temperatures.
556 Additionally, a *Serine threonine- protein kinase pim3*, an enzyme involved in the regulation of
557 cell transport and survival, which prevents apoptosis by inducing the release of the anti-apoptotic *Bcl2*
558 mentioned before (Cross et al., 2000) was also overexpressed at 7˚C, whereas a *Serine threonine-*
559 *protein phosphatase 6,* with opposite function to the kinase enzyme (Cross et al., 2000), was down-
560 regulated at 22˚C. Another interesting finding is the opposite pattern of gene expression found
561 between experiments for the *Wsc domain-containing protein 1* (down-regulated at 7˚C and up-
562 regulated at 22˚C) (Supplementary Information S3). Different members of the *Wsc* family are
563 identified as putative receptors of stress and required for the heat shock response and the maintenance
564 of cell wall integrity in yeasts (Lodder, Lee & Ballester, 1999). The *Wsc* members are upstream
565 regulators of other serine-threonine kinases, the protein kinase C1 (PKC1) and mitogen-activated

566 protein kinase (MAPK), which can promote apoptosis (Lodder et al., 1999; Cross et al., 2000). The
567 differential expression of these molecules between control conditions, 7˚C, and 22˚C, evidences the
568 different regulation systems of apoptosis and control of cell damage at different temperatures in *A.*
569 *lixula*.

570 Previous experiments on echinoderms demonstrated the effect of thermal stress on the
571 immune capacity of coelomocytes, being this effect greater at higher than lower temperatures in the
572 analysed species (the sea cucumber *Apostichopus japonicus*, Wang, Yang, Gao & Liu, 2008).
573 However, in *A. lixula*, it was the lowest temperature the one that triggered a higher immune response
574 in terms of gene expression. The *echinoidin*, *senescence associated-gene*, *cytohesin-like* and *tripartite*
575 *motif-containing protein 3* (TRIM) (Supplementary Information S3) involved in the infection response
576 and/or pathogen-recognition process against bacteria, fungi and viruses (Smith et al., 2006; Ozato,
577 Shing & Chang, 2008) were up-regulated at 7ºC. In addition, the gene *interleukin-17* (Supplementary
578 Information S3) which is a cytokine inducing and mediates proinflammatory responses in metazoans
579 and stimulates phagocytosis in echinoderms (Beck et al. 1993), was also up-regulated at 7˚C. None of
580 these immune genes were, however, activated (or, when detected, were down-regulated) at the highest
581 experimental temperature (e.g. TRIM), suggesting no immune response at 22˚C.
582

583 The differentially expressed genes for the low and high temperature experiment were
584 associated to different GO categories that provide additional information. These GO categories
585 summarise the most significant biological processes, cellular components, and molecular functions
586 that were up- and down- regulated during the experimental response in *A. lixula*. For the high thermal
587 stress experiment, we could only recover GO terms of three transcripts, and therefore, there is limited
588 information to reach conclusions on the GO categories for this experiment. However, we detected the
589 down-regulation of two interesting GO terms, the "Notch signaling pathway" with the associated gene
590 *neurogenic locus notch* (*Notch1*), and the "integral component of membrane" with the associated gene
591 encoding a Notch ligand, the *delta protein.* Notch is a cell signaling system calcium-dependent
592 involved in different functions including cell differentiation, proliferation and apoptosis. In general,

593 Notch inhibits apoptosis and induces cell proliferation but, *in vitro* studies, using different cell
594 lineages, showed that hyperthermia reduced *Notch1* expression and apoptosis in some cell lineages,
595 whereas a opposite pattern was obtained in other cell lineages (Basile et al., 2007). Therefore, the
596 effect of the Notch down-regulation at high temperatures in coelomocytes is not completely clear, but
597 it suggest the existence of an alternative pathway of apoptosis under thermal stress.

598 Among the GO terms up-regulated during cold exposure that add further information we
599 found the "Tyrosine metabolism" term, which is related to cell protection against stress, including the
600 up-regulation of HSPs, cytoskeletal stabilization and apoptosis decrease (Baird, Niederlechner, Beck,
601 Kallweit & Wischmeyer, 2013). This major GO term also includes the subordinate "Positive
602 regulation of apoptotic process", which can induce apoptosis when protein refolding by HSPs is not
603 efficient enough. The induction of HSPs during thermal stress can considerably increase the energy
604 demand in cells (Tomanek 2010; Dong, Yu, Wang & Dong, 2011). This increased energy demand is
605 reflected in the over-representation of the GO category "ATP hydrolysis", a catabolic process that
606 releases energy previously stored in the form of ATP, and the up-regulation of the *V-type proton*
607 *ATPase* gene (see Supplementary Information S3), a proton pump found within the
608 "proton−transporting V−type ATPase, V0 domain" term. Likewise, the terms "Protein folding" and
609 "Protein transport", the last one subordinate to the "ATP hydrolysis" category, are linked to protein
610 transport to the Sarcoplasmic reticulum for folding reaction to avoid protein denaturalization by HSPs.
611 Hence, the "Sarcoplasmic Reticulum" category, a key organelle involved in the thermal stress
612 response that ensures that misfolded proteins are directed towards a degradative pathway to the central
613 cytoplasmic proteolytic machinery (Malhotra & Kaufman, 2007), was also over-represented at 7ºC.
614 Actually, the induction of expression of *Hsp70s* has been directly associated to the accumulation of
615 unfolded proteins in the sarcoplasmic reticulum (Rachel, Tyson & Stirling, 1997; Rao et al., 2002),
616 which are later eliminated if refolding fails by retrograde transport across the reticulum membrane
617 (Kostova & Wolf, 2003). Other minor up-regulated GO terms, at the biological process and molecular
618 function, were "oxidation-reduction processes" (1 Go term) and "oxidoreductase activitiy" (2 Go
619 terms). These terms suggest that low temperature affects the intracellular redox state in coelomocytes.

620 Among the down- regulated GO terms at the 7˚C treatment we found “Neurotransmitter
621 transport” with the associated differentially expressed genes *Creatine transporter* and *Trafficking*
622 *protein particle complex subunit 2 protein*. The *Creatine transporter* is essential for normal brain
623 function in humans and tissues with high energy demands because, together with other molecules,
624 maintains ATP levels (Christie, 2007). The down-regulation of these genes and pathways could be a
625 potential response to energy competition with the induction of HSPs during thermal stress. The 7˚C
626 treatment also seemed to inhibit nuclear replication, as represented by the down-regulation of the
627 “nuclear origin of replication recognition complex” and “DNA replication” terms, among others. The
628 origin recognition complex is a ATP-dependent system that, among other factors, enables the initiation
629 of DNA replication in eukaryotic cells (Li & Stillman, 2013). Cells under stressful conditions must
630 prevent cell division in favour of protective funtions (Jonas, Liu, Chien & Laub, 2013), as well as to
631 avoid entering in a new DNA replication cycle if there is DNA damage (Lee et al., 2009). We also
632 found down-regulation of the “Intracellular signal transduction” term, with the subordinate “cell redox
633 homeostasis” and “smoothened signalling pathway” terms, and the “protein O-linked glycosylation”
634 term. Smoothened is a key transmembrane protein involved in a critical cell-to-cell communication
635 system for tissue homeostasis. Glycosylation, on the other hand, is one of the most common post-
636 transcriptional modifications during protein biosynthesis, which contributes to increase protein
637 solubility and stability against proteolysis, and can also be involved in their correct folding (Shental-
638 Bechor & Levy, 2008). Hence, the down-regulation of these last two terms reflects the potential
639 negative effect of low temperatures on protein biosynthesis and stabilization, and homeostasis control
640 in coelomocyte cells.

641 In summary, our results based on RNA-seq analyses of the whole transcriptome of
642 coelomocytes in *A. lixula* show that this sea urchin, or at least this NW Mediterranean population
643 (Wangensteen et al. 2012; Pérez-Portela et al. 2019), displays strong gene expression changes in
644 response to the cold treatment, with activation of many genes whose functions could be related to
645 stress responses in the form of chaperone production, apoptosis regulation, ATP-associated genes,
646 enhancement of the immune system and redox processes, and down-regulation of gene pathways

647 related to protein biosynthesis and DNA replication. Nevertheless, contrary of that found in other
648 studies (e.g. Gleason & Burton, 2015; Zhu et al., 2016) no activation of genes encoding antioxidant
649 enzymes was detected in our experiments. As we initially expected, a markedly lower response is
650 found in the warm treatment, with no activation o deactivation of the previously mentioned pathways,
651 with the exception of the apoptosis regulation. Although some caution is needed, as we have
652 characterized transcriptional changes and not protein levels, the differential patterns found in these
653 genes strongly indicated that sea urchins are more stressed under lowered experimental temperatures.

654 We acknowledge that we have tested only acute thermal conditions, without any progressive
655 acclimation. This is an unrealistic scenario but was chosen to elicit a short-term measurable response.
656 This response was much more marked against lower than higher temperatures, which indicates
657 potential to compensate for cold stress. Future research should investigate a wider panoply of
658 temperature regimes combined with acclimation periods. However, our results indicate that *A. lixula*
659 might require energy expenditure to withstand the stress associated with low temperatures, while it
660 does not undergo relevant transcriptional changes when exposed to warm temperatures. This is
661 coherent with the notion of a thermophilous species living near the colder limit of its physiological
662 tolerance, as found also when analysing reproductive and larval features (Wangensteen et al., 2013a,
663 2013b).

664 It has been suggested that the tropicalization of NW Mediterranean can lead to a shift in
665 dominance between the temperate common sea urchin *Paracentrotus lividus*, which will suffer from
666 warming temperatures, and the thermophilous black sea urchin *A. lixula* (Gianguzza et al., 2011,
667 Wangensteen et al., 2013a,b, Carreras et al., 2020). Such a shift can have drastic ecological impacts, as
668 both species are conspicuous engineer species shaping benthic communities (Bulleri et al., 1999,
669 Bonaviri et al., 2011). Specific biological and genomic studies are needed to understand the adaptive
670 capabilities of *A. lixula* to ongoing warming, but our results add to the available evidence that colder
671 rather than warmer temperatures may be a limiting factor for *A. lixula*. The absence of clear signs of
672 stress at warm temperatures in adults of *A. lixula,* together with information on larvae development
673 and gonad maturation (Wangensteen et al., 2013a and 2013b), support the hypothesis of the positive

674 effect of winter warming on the species' reproduction output and larval survival. The ongoing
675 expansion of the species across the littoral coast of the Mediterranean, with the concomitant impacts
676 of its grazing activity on littoral communities, may be exacerbated in the near future by rising winter
677 temperatures in the NW Mediterranean.

678

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685

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1135 DATA ACCESSIBILITY
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1137 The *De novo* assemblies, RSEM, annotation and DEseq files are available at Mendeley Data
1138 doi.org/10.17632/5673n552yj.1.
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1141 AUTHOR CONTRIBUTIONS
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1143 All authors contributed to the design of this study and were involved in the aquarium
1144 experiments. RPP, AR, XT and OSW analysed the data. RPP wrote the first draft of the
1145 manuscript and created figures and tables. XT, AR and CP contributed to improve the first
1146 draft, and all authors revised the final version of the manuscript.
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1149 FIGURE LEGENDS
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1152 Comparison between Control condition (CT at 13°C) and temperature at 7°C (CONTROL *vs* T7), and
1153 Control and 22°C (CONTROL *vs* T22). Red crosses indicated replicates lost during the development
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1178 [**1**= multicellular organism development; **2**= positive regulation of GTPase activity; **3**= regulation of
1179 ARF protein signal transduction; **4** single−organism cellular process**;** **5**= DNA replication; **6**=
1180 polyamine biosynthetic process; **7**= histone H3 deacetylation; **8**= DNA−templated transcription
1181 initiation; **9**= microtubule−based movement; **10**= mitochondrion organization; **11**= microtubule motor
1182 activity; **12**= calcium ion binding; **13**= pantothenate biosynthetic process; **14**= microtubule−based

1183 process; **15**= RNA secondary structure unwinding; **16**= oxidation-reduction process; **17**= heterocyclic
1184 compound binding; **18**= organic cyclic compound binding; **19**= sequence−specific DNA Binding; **20**=
1185 poly(A) RNA Binding; **21**= heme binding; **22**= methyltransferase Activity; **23**: protein kinase activity;
1186 **24**= monooxygenase activity; **25**= oxidoreductase activity; **26**= peptidyl−prolyl cis−transisomerase
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1194 Processes, Cellular components, and Molecular functions. The size of the rectangles reflects the
1195 log2foldchage associated to the differentially regulated categories.
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Reference	Reads	Genes	Transc.	N50	N20	Blast hit	GC%	M_L	Avg_L	Mb
CT+T7	341,735,712	151,418	211,456	1,102	2,960	41,429	41.68	345	668.6	141.5
CT+T22	351,275,576	151,278	215,362	1,114	2,976	38,691	41.41	345	671.1	147.4

1198
1199 Table 1. General statistics of the reference assemblies CT_T7 and CT_T22 of *A. lixula*. Total number
1200 of trimmed reads assembled (Reads), number of genes, number of transcripts (Transc.: including
1201 genes and isoforms collapsed into genes), parameters N50 and N20, number of transcripts with Blast
1202 hit against proteins of metazoans, percentage of GC (GC%), median transcript length (M_L), average
1203 transcript length (Avg_L), and number of assembled bases expressed as Mb.
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Treatment	Replicate	Gender	Nº Reads
7ºC	1	F	74,582,590
	2	M	40,645,290
	3	F	83,661,578
	4	M	60,222,882
	5	M	140,478,920
Control	1	F	41,330,158
	2	M	84,053,170
	3	M	23,074,766
	4	F	59,854,444
	5	F	26,843,788
	6	M	48,723,838
22ºC	1	F	50,432,902
	2	M	36,718,372
	3	F	139,884,462
	4	M	103,939,816
	6	M	87,695,436

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1213 Table 2. Treatment and replicate, gender and number of trimmed reads used for differential expression
1214 analyses. Note that replicates 6 and 5 from 7ºC and 22ºC, respectively, are missing due to low quality
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Eukarya						
Reference	**C**	**S**	**D**	**F**	**M**	**Groups searched**
CT+T7	290 (95.7%)	203 (67%)	87 (28.7%)	12 (4%)	1 (0.3%)	
CT+T22	286 (94.4%)	206 (68%)	80 (26.4%)	16 (5.3%)	1 (0.3%)	303

Metazoa						
Reference	**C**	**S**	**D**	**F**	**M**	**Groups searched**
CT+T7	924 (94.5%)	618 (63.2%)	306 (31.3%)	45 (4.6%)	9 (0.9%)	
CT+T22	925 (94.6%)	603 (61.7%)	322 (32.9%)	42 (4.3%)	11 (1.1%)	978

1229
1230 Table 3. Number and percentage of BUSCO groups recovered in the searches against the eukaryotic
1231 and metazoan databases. Complete BUSCOs (C), complete and single-copy BUSCOs (S), complete
1232 and duplicated BUSCOs (D), fragmented BUSCOs (F), and missing BUSCOs (M).
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Experiment	DE transcripts	Blast hit	GO term
Control vs T7	1181	445	84
Control vs T22	179	35	3

1244
1245 Table 4. Differentially expressed (DE) transcripts between treatments. Number of DE transcripts
1246 between treatments for each experiment, number of DE transcripts with blast hit against metazoan
1247 proteins per experiment, and number of DE transcripts with an associated GO term per treatment.
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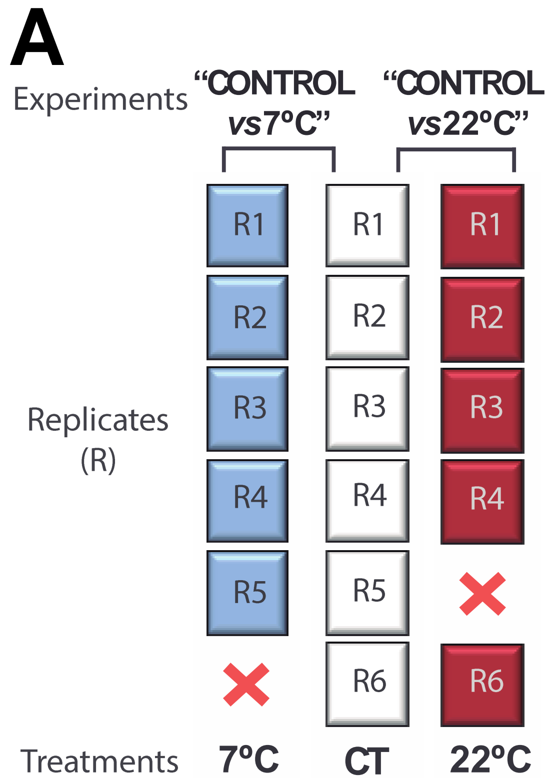

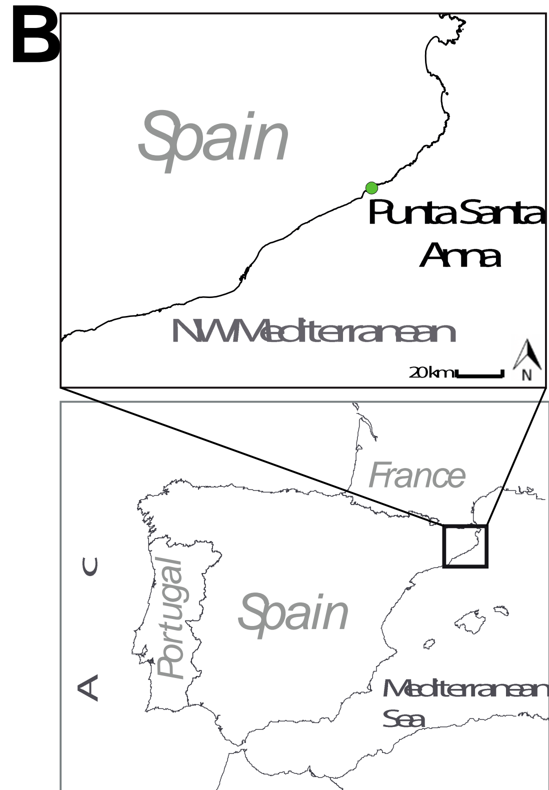

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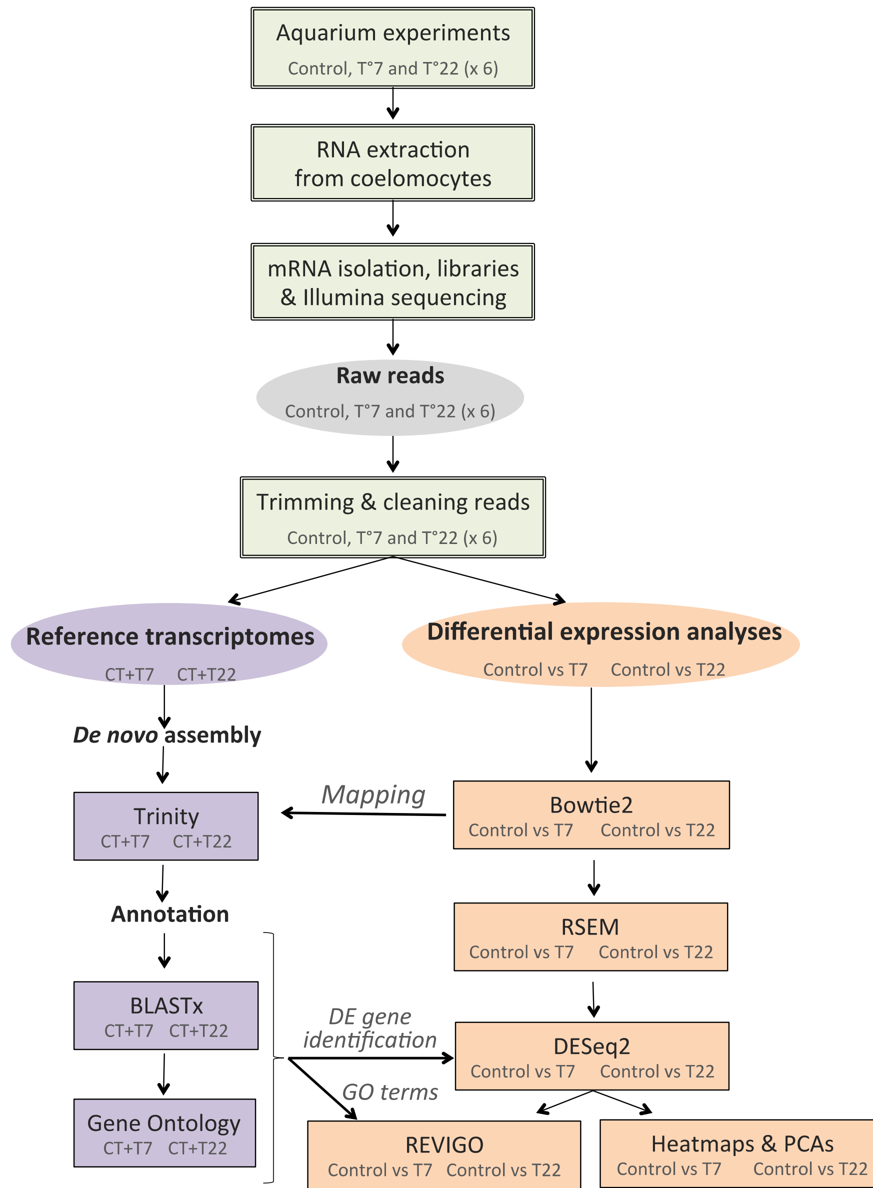

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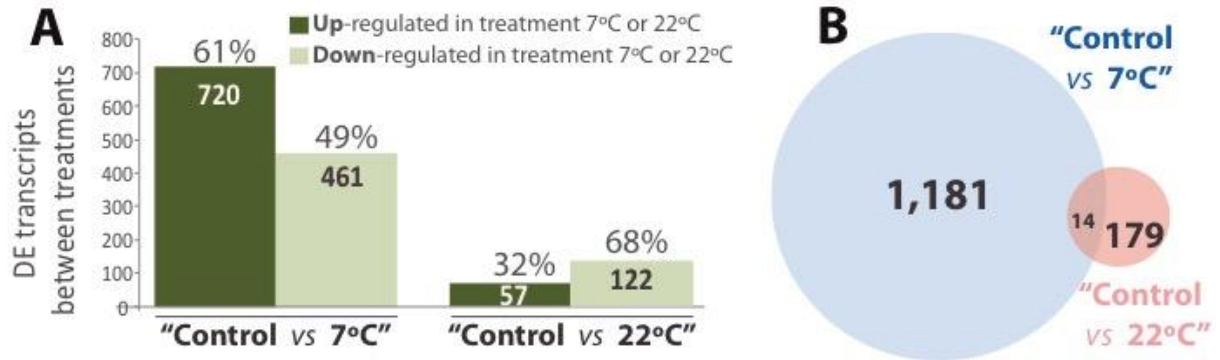

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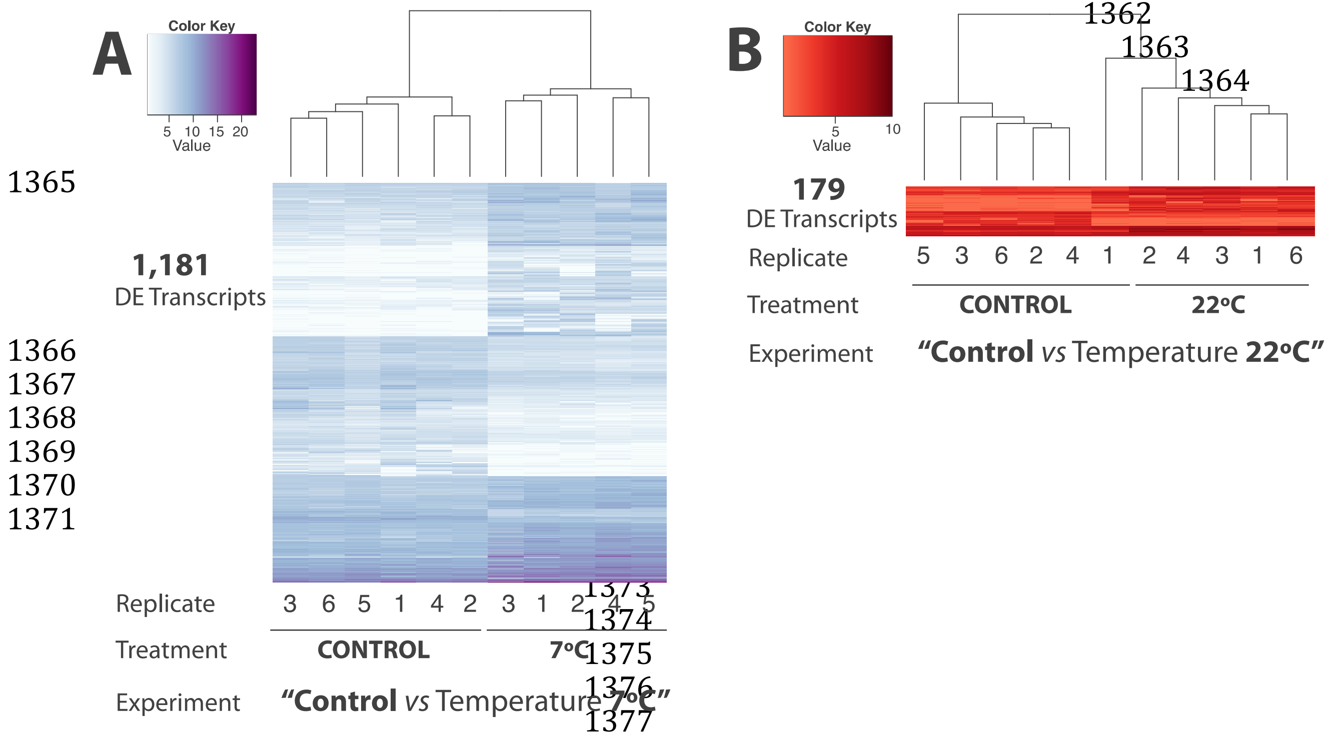

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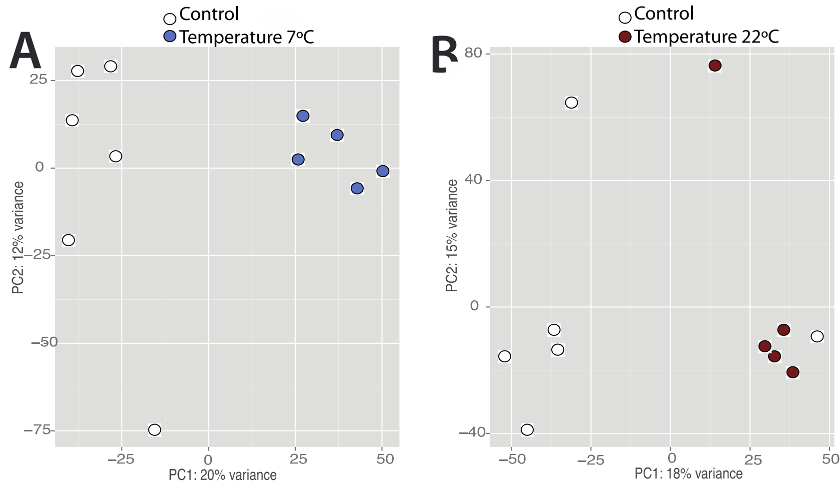

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1478 condition.
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