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

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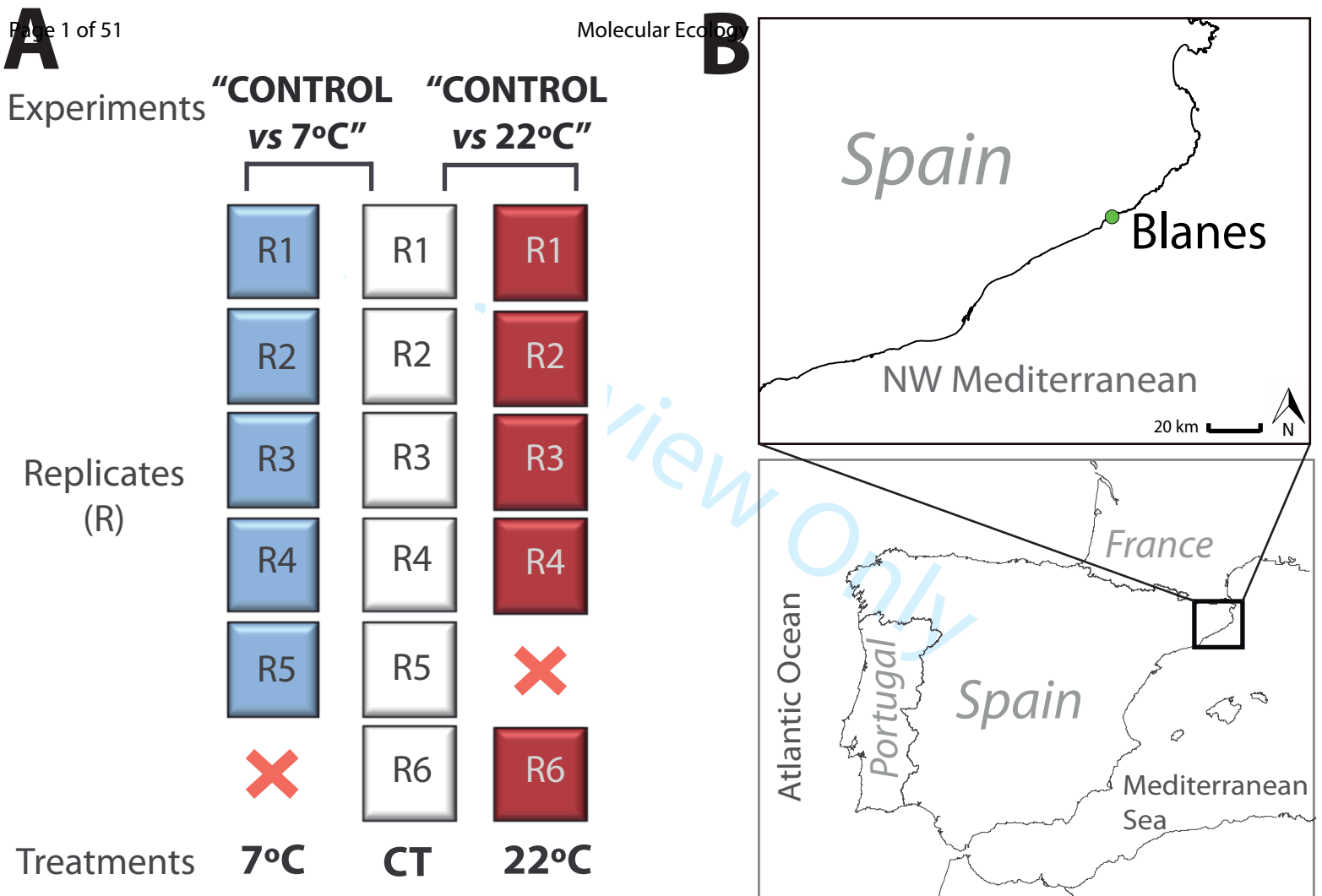

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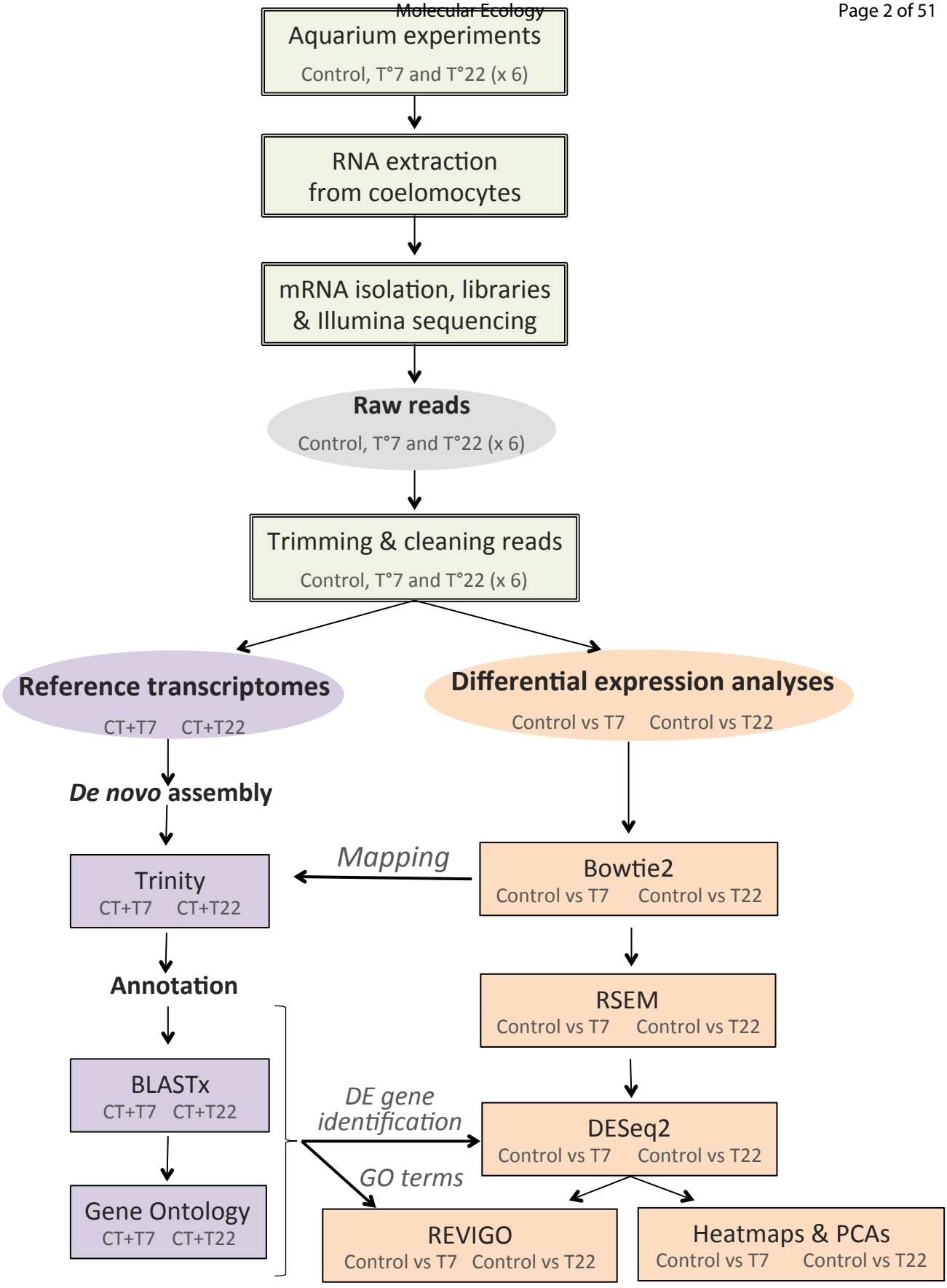
Aquarium experiments
Control, T°7 and T°22 (x 6)
RNA extraction from coelomocytes
mRNA isolation, libraries & Illumina sequencing
Raw reads
Control, T°7 and T°22 (x 6)
Trimming & cleaning reads
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Heatmaps & PCAs
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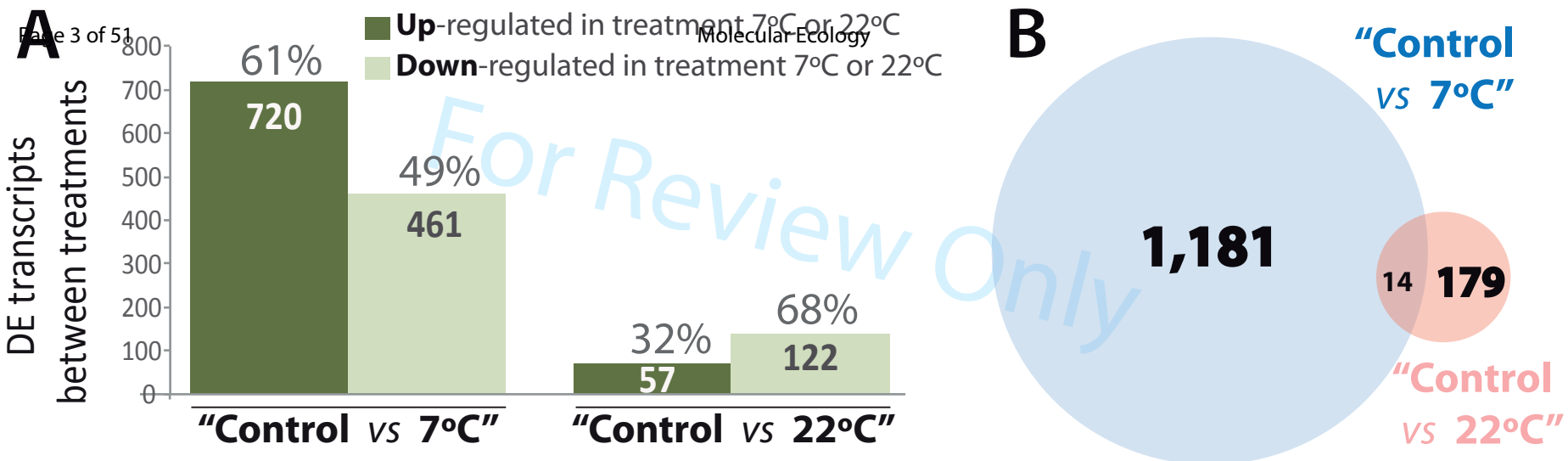

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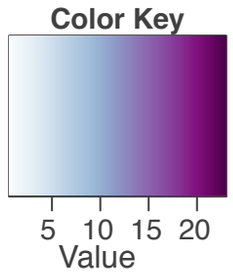
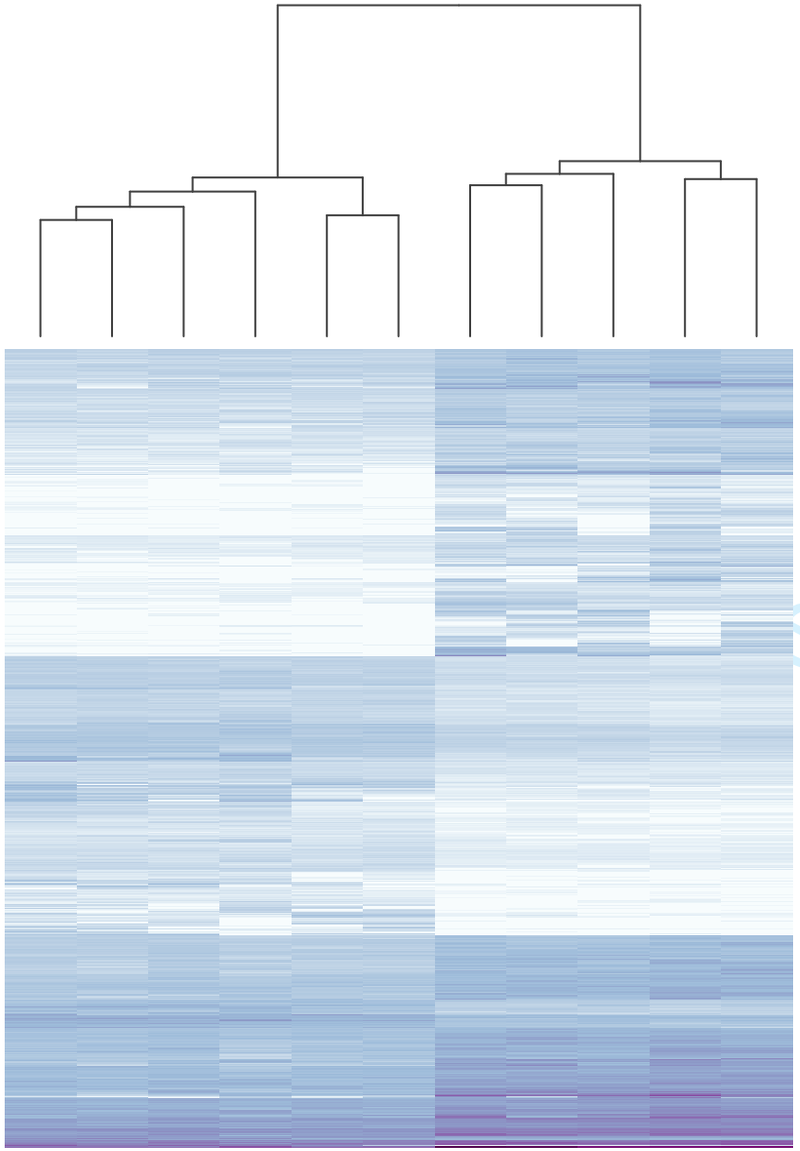




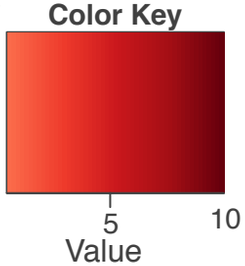
1,181
DE Transcripts

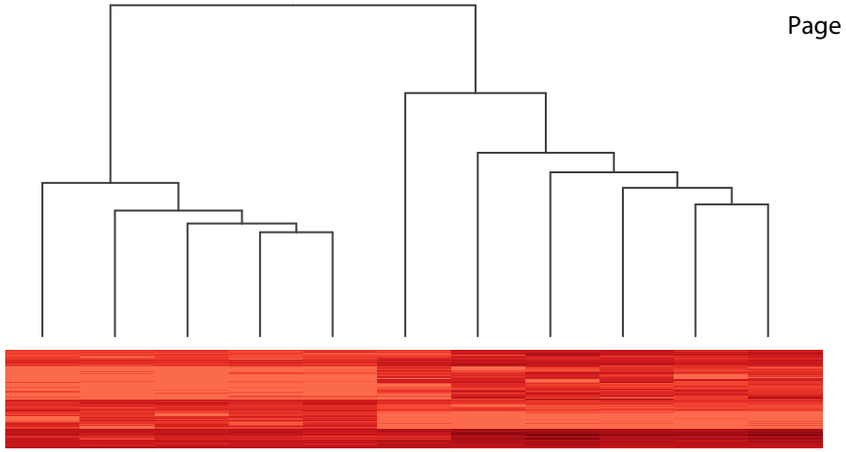
Replicate 3 6 5 1 4 2 3 1 2 4 5

Treatment **CONTROL** **7°C**

Experiment **"Control** *vs* Temperature **7°C"**

B





179
DE Transcripts

Replicate 5 3 6 2 4 1 2 4 3 1 6

Treatment **CONTROL** **22°C**

Experiment **"Control** *vs* Temperature **22°C"**

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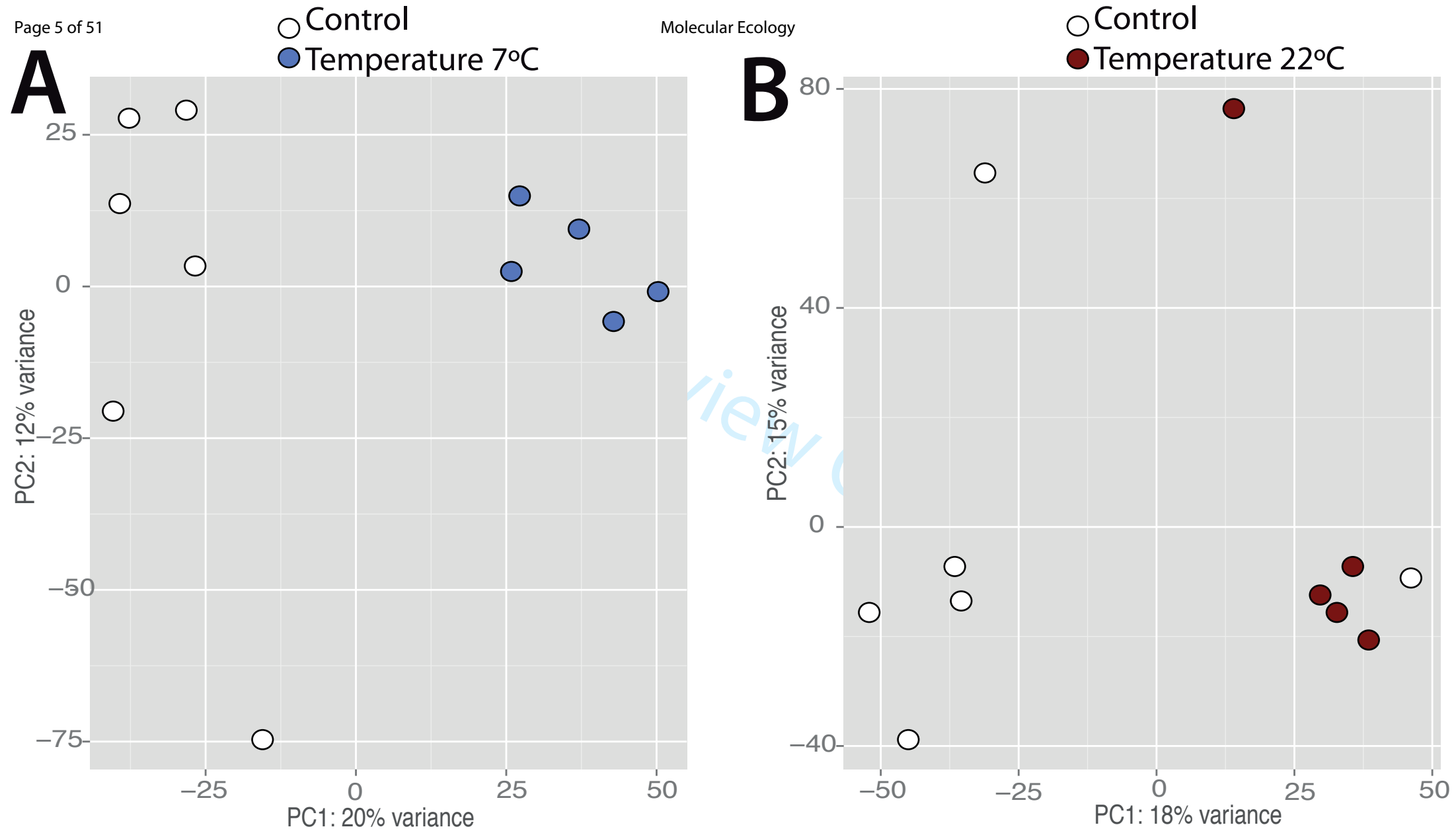
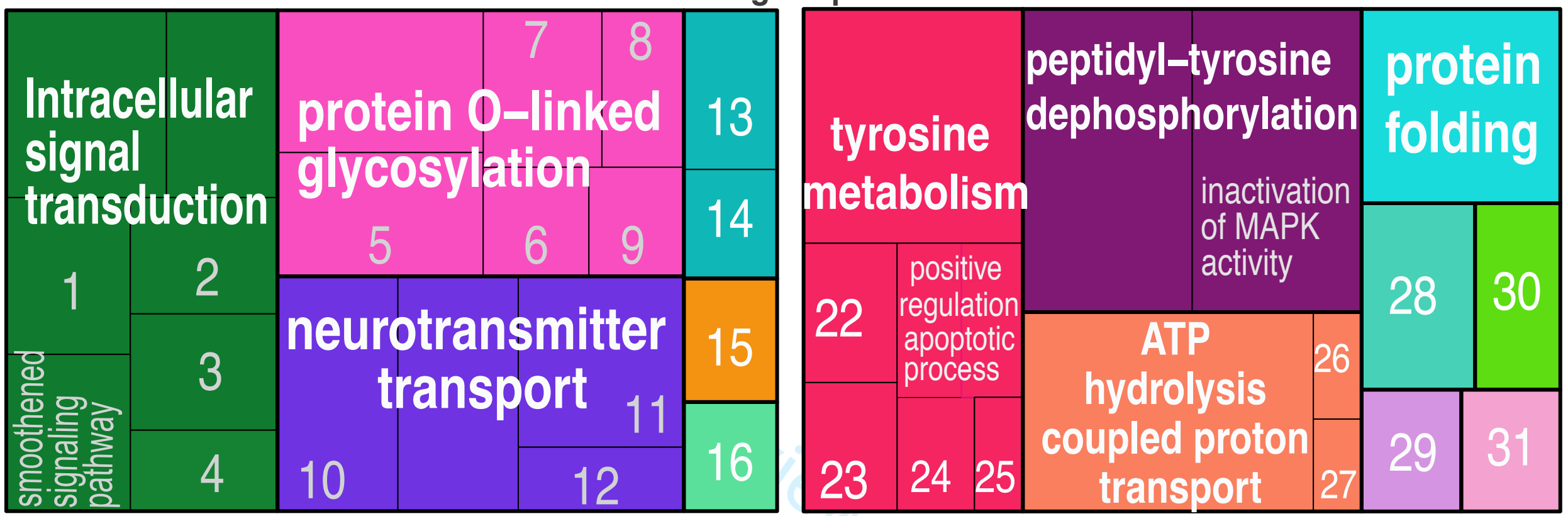

A
Control
Temperature 7°C
25
0
−25
−50
−75
PC2: 12% variance
−25
0
25
50
PC1: 20% variance
B
Control
Temperature 22°C
80
40
0
−40
PC2: 15% variance
−50
−25
0
25
50
PC1: 18% variance

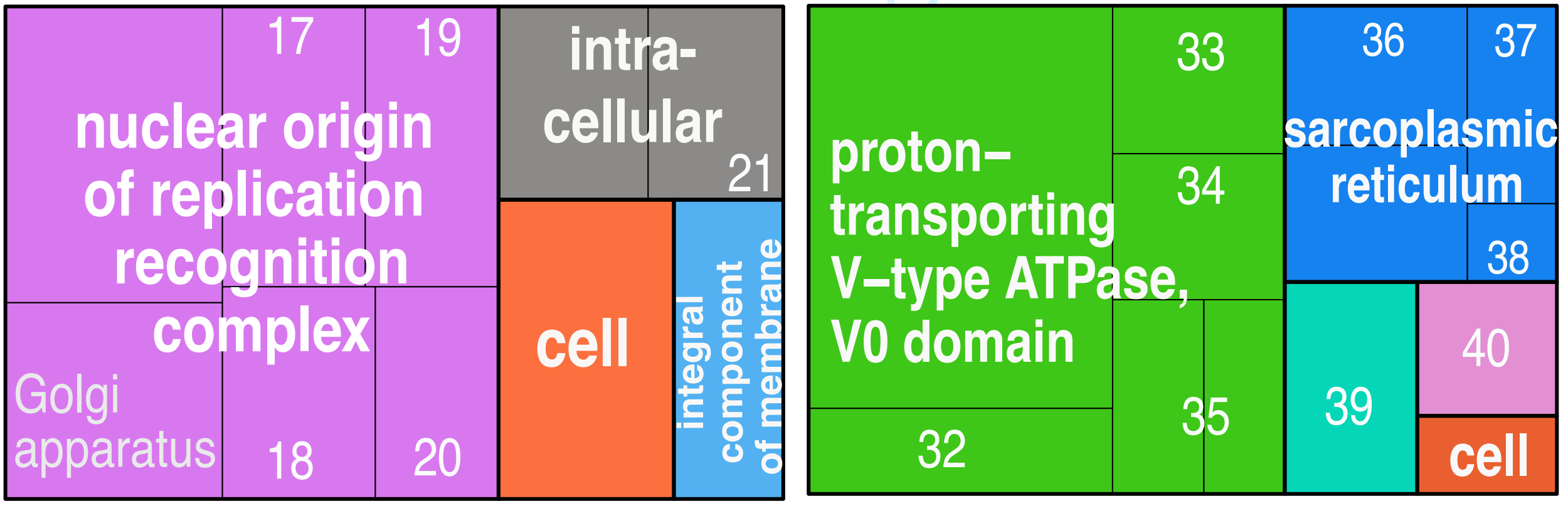
DOWN-REGULATED IN TEMPERATURE 7ºC

UP-REGULATED IN TEMPERATURE 7ºC

Biological process



Cellular component



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DOWN-REGULATED IN TEMPERATURE 22°C

Biological process

Notch signaling pathway

multicellular organismal development

Cellular component

integral component of membrane

membrane

SAGA–type complex

Molecular function

calcium ion binding

calcium ion binding

metal ion binding

protein binding

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

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Running title: Transcriptomic response to thermal changes

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ABSTRACT

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

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

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INTRODUCTION

Predicting organismal responses to environmental shifts is one of the main priorities of contemporary ecology (Calosi et al., 2017; King, McKeown, Smale, & Moore, 2018; Donelson et al., 2019). During the last few decades, scientific studies have linked global warming, characterised by both an increase in mean temperatures and frequency of heat waves (Jordà, Marbà & Duarte 2012; Oliver et al., 2018), to detrimental impacts on marine systems at different biological levels. These impacts can involve disruption of the whole ecosystem functioning, resulting from changes in food-wed dynamics and in ecosystem productivity, together with biodiversity loss (see Smale et al., 2019; Stillman, 2019), to more specific effects. On the other hand, mean temperature increases and heat waves also result in a number of lethal and sub-lethal effects on particular species and/or populations, including coral reef bleaching (Hughes et al., 2017), alteration of animal migration behaviour, and shifts of marine taxa distribution patterns, among many others (e.g. Hoegh-Guldberg & Bruno, 2010; Deutsch, Ferrel, Seibel, Pörtner & Huey, 2015; King et al., 2018).

Under sub-lethal extreme temperature, stress is likely to occur (e.g. Buckley & Huey, 2016), and organisms have developed different molecular and cellular mechanisms to maintain physiological performance and cell homeostasis (Pörtner, 2002; Buckley & Huey, 2016). Thermal responses include changes in expression patterns of stress-responsive genes, including genes that regulate metabolism (e.g. Xu, Zhou & Sun, 2018; Zheng et al., 2019), oxidation-reduction processes (e.g. Gleason & Burton, 2015; Xu et al., 2018; Zheng et al., 2019), protein folding repair systems (Gleason & Burton, 2015; Kim, Kim, Choi & Rhee, 2017; Xu et al., 2018; Zheng et al., 2019), and cell cycle (Zhu et al., 2016; Xu et al., 2018; Zheng et al., 2019). Among the best-characterised molecules involved in stress response are the Heat Shock Proteins (HSPs) (e.g. Feder & Hofmann, 1999; Tomanek, 2010; Kim et al., 2017). HSPs are highly conserved molecular chaperones that help proteins' folding and transport across cell membranes during non-stressful conditions. They also refold and stabilise denatured proteins under different conditions of stress (e.g. Matranga, Toia, Bonaventura & Müller, 2000; Di Natale et al., 2019). Although HSPs' function is well-known in some marine invertebrates, additional

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knowledge on the involvement of other molecular pathways, such as antioxidant genes, apoptosis-associated and immune-associated genes, is needed to uncover other relevant mechanisms involved in thermal stress responses in ecologically relevant species (Gleason & Burton, 2015; Zhu et al., 2016). One relatively recent approach to investigate rapid organismal responses to environmental perturbations, to identify potential physiological networks, and to discover candidate genes and isoform variants involved in their responses, is to explore the whole transcriptional profiles using RNA-seq techniques (e.g. Zhu, Zhang, Li, Que & Zhang, 2016; Evans, Pespeni, Hofmann, Palumbi & Sanford, 2017; Xu et al., 2018). Although the relationship between mRNA transcript abundance and protein abundance is still not clear (Feder & Walser, 2005), some studies have shown a correlation between these two variables (Maier, Güell & Serrano, 2009). Changes in gene expression are considered to be sensitive indicators of stress and potential predictors of organismal physiology under experimental conditions (Feder & Walser, 2005; Buckley, Gracey & Somero 2006; Schoville, Barreto, Moy, Wolff & Burton, 2012).

Among marine ecosystems, one the most impacted seas in the world is the Mediterranean (Lejeusne, Chevaldonné, Pergent-Martini, Boudouresque & Pérez, 2010; Coll et al., 2010). This sea holds high levels of biodiversity while at the same time it is subjected to intense anthropogenic pressures (Claudet & Fraschetti, 2010; Templado 2014), which interact with the ongoing global warming (Francour, Boudouresque, Harmelin, Harmelin-Vivien & Quignard, 1994; Jordà et al. 2012). During the last three decades, sea surface temperature (SST) has risen in the Mediterranean at a rate of 0.25 – 0.65°C decade^{-1} in the western and eastern basins, respectively (Marbà, Jordà, Agustí, Girard & Duarte, 2015). High-resolution ocean models, considering a diversity of potential climate change scenarios, have projected in all cases a significant increase in SST by the end of the century (see Somot, Sevault & Déqué, 2006; 2008; Parry, Canziani, Palutikof, Van Der Linden, & Hanson, 2007; Shaltout, & Omstedt, 2014). A warmer Mediterranean represents a challenge for most mediterranean taxa, reflected in sub-lethal effects linked to behavioural and physiological responses (e.g. Anestis, Lazo, Pörtner & Michaelidis 2007; Prusina et al., 2014), lethal outcomes, including mass mortality events associated to heat waves (e.g. Cerrano et al., 2000; Coma et al., 2009; Garrabou et al., 2009),

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

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

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

The aim of this study is to explore the short-term transcriptional response to thermal changes among individuals of the subtropical sea urchin *A. lixula*. We set three specific objectives for our study: a) To quantify and compare transcriptional responses to both high and low temperature treatments in *A. lixula* under experimental conditions, b) To identify some of the most important candidate genes involved in rapid thermal responses in sea urchins, and c) To determine the existence

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of common genes involved in responses to increasing and decreasing temperatures.

Many studies on global warming focus on the negative effect of rising temperatures, but in this study, we worked under the hypothesis that *A. lixula* will experience higher stress when subjected to low rather than to high temperatures. Based on previous transcriptional information from marine invertebrates under thermal stress (e.g. Gleason & Burton, 2015; Zhu et al., 2016), we also expect changes of expression patterns in different gene pathways during our temperature treatments, including genes encoding HSPs, apoptosis and anti-apoptosis mechanisms, ATP-associated genes due to an increase of energy demand to maintain cell homeostasis, antioxidant genes since extreme temperatures can increase cells' oxidative stress, and immune-associated genes (Xu et al., 2018). The information obtained here will be relevant to understand the ecophysiological patterns of sea urchins exposed to thermal changes. We also discuss the significance of our findings for the foreseeable ecological spread of this keystone species in the Mediterranean.

MATERIAL AND METHODS

Sea urchin collection

Adult specimens of *A. lixula* were collected by SCUBA diving in December 2012 from the shallow subtidal population (5-8 m depth) of Punta Santa Anna, in the locality of Blanes (41°40′22.47″N, 2°48′10.81″E, North-western Mediterranean; Figure 1). Specimens were quickly transported to the laboratory (less than 2 Km away) in a cooler with seawater and oxygen tablets to keep stress induced by land transportation to a minimum. Experiments were performed in the LEOV (Laboratory of Experimentation with Living Organisms) facility of the Centre for Advanced Studies of Blanes (CEAB), equipped with an open system of running seawater coming directly from a sea intake. Once in the laboratory, sea urchins were measured with callipers and left to adjust for 48 hours in a common chamber with airflow and flow-through running seawater at 13˚C, which was the sea temperature in Blanes at the collection time. During these 48 hours animals had rocky surfaces

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189 available for grazing.

190

191 *Experimental design*

192

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

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

Our experimental design for transcriptomic analysis consisted of two different experiments: A "Low temperature" experiment comparing the control condition at 13˚C ± 1˚C and experimental condition at 7˚C ± 0.5˚C, hereafter named as "Control vs T7", and a "High temperature" experiment comparing the control condition at 13˚C ± 1˚C and experimental condition at 22˚C ± 0.5˚C, hereafter named as "Control vs T22" (see Figure 1). Samples used as control condition were the same for both experiments, since all treatments were run at the same time and laboratory. After the acclimation period of 48 hours, each sea urchin was placed in an independent aquarium to avoid interactions among specimens. Each aquarium had constant airflow and the seawater temperature was set at the required temperature (13ºC, 7ºC or 22ºC) prior to adding the sea urchins. Temperature of the aquaria was controlled with HOBO loggers (one per aquarium). Aquaria with different treatments were randomly allocated across the wet-lab space to avoid any bias related to their spatial distribution. Animals were not fed during the 20 h of the experimental time, and seawater pH (8.1) was monitored

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during the experiments. Eight different replicates (specimens) per treatment were included, although for gene expression analyses only six of them were processed. The sample size of 8 was used to ensure an even proportion of sexes in the specimens analysed (since sex determination can be only performed *a posteriori* after dissection), and indeed we processed for transcriptomic analyses 3 males and 3 females per treatment. After the 20 hours of treatment, sea urchins were removed from the aquaria, quickly dissected under RNAase free conditions, and coelomocyte fluid collected and processed as explained in the next section.

For sex determination we used histological techniques. One gonad per individual was obtained and preserved in 4% formaldehyde. Gonad samples were washed in distilled water, dehydrated, embedded in paraffin, cut in 5 µm sections using a Microm HM325 Microtome, and stained in haematoxylin–eosin as described in Wangensteen et al. (2013b) and Garcia-Cisneros et al. (2017). Sex was then determined under the optical microscope.

Coelomocytes collection and RNA sequencing

Coelomocytes consist of several cell types contained in the coelomic fluid and are immune effectors in echinoderms (Matranga et al., 2000; Smith et al., 2018). They have been used as biomarkers of stress due to their prompt response to changing environmental conditions (Matranga et al., 2000, Matranga, Bonaventura & Di Bella, 2002; Matranga et al., 2005; Pinsino et al., 2008) that can reduce the protective capacity of these cells and rapidly induce activation of the heat shock proteins expression (Matranga et al., 2000; Pinsino et al., 2008). Additionally, these cells showed higher thermal response capacity than other tissues in sea urchins (e.g., digestive tissues, Gonzalez et al., 2016), and protocols for extraction of high quality RNA and high throughput sequencing have been developed for this tissue type in *A. lixula* (Pérez-Portela & Riesgo 2013; Pérez-Portela et al., 2016).

Five millilitres of the coelomic fluid of each specimen (a total of 18 specimens; six per treatment) were collected using a sterile syringe inserted through the peristomic membrane, taking

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care not to puncture the gut. The fluid was then centrifuged (50x g), and all fresh cellular components (coelomocytes) gathered and quickly embedded in TRizol reagent (Invitrogen, www.invitrogen.com). Total RNA was directly extracted from coelomocytes following a protocol previously optimised for this species (Pérez-Portela & Riesgo 2013 and Pérez-Portela et al., 2016). Integrity of total RNA and potential DNA contaminations were initially evaluated by visualizing the 28S rRNA and 18S rRNA bands into a 1% agarose gel in 1x TAE Buffer. Concentration of the RNA extracts was assessed in a Hellma spectrophotometer (Hellma Analytics), and total RNA extracts were also run in an Agilent 2100 Bioanalyzer (Agilent Technologies) at the Scientific and Technical Services of the University of Barcelona for quality measurements. High quality RNA (RINs over 8.5) samples were sent to the National Centre of Genomic Analyses of Barcelona (CNAG) for mRNA isolation, cDNA library construction, normalization and sequencing.

Isolation of mRNA and cDNA library preparation for each of the 18 specimens were performed using the Illumina TruSeq RNA Sample Prep Kit (Illumina, Inc.) following the manufacturer's recommendations, with an input of 800-900 ng of mRNA, and average insert size of the libraries of 300 bp. Quality and concentration of the 18 cDNA libraries was controlled with Ribogreen Assays in a NanoDrop 3300™ Fluorospectrometer (Thermo Fisher Scientific, www.thermofisher.com). The 18 libraries (6 per treatment) were multiplexed with Illumina barcodes, and 5 libraries per lane were sequenced on an Illumina HiSeq2000 Sequencer, generating 101 base paired-end reads. The 18 libraries from different treatments were randomised across Illumina lanes.

Sequence processing and de novo *assembly*

The software FASTQC v. 0.10.0 (www.bioinformatics.babraham.ac.uk) was used to visualise and measure the quality of the raw reads generated in the HiSeq2000. Adapters and bases with low quality (phred scores <33) were trimmed off, and a length filter was applied to keep only sequences of >25 bases using TrimGalore v. 0.2.6 (www.bioinformatics.babraham.ac.uk). High-quality reads were

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re-screened in FASTQC to ensure a good quality of the samples after trimming. A basic scheme of the most important steps of our pipeline is presented in Figure 2.

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

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

Blast results against Metazoa served as a database for annotation of transcripts differentially expressed between treatments (see below). Moreover, Blast results of the assemblies were used to retrieve Gene Ontology (GO) terms with BLAST2GO (Conesa et al., 2005) under different categories: biological processes, cellular component and molecular function, which are hierarchically organized into different levels (see Figure 2). The completeness of the reference transcriptomes was assessed with BUSCO (Benchmarking Universal Single-Copy Ortholog) against the eukaryotic and metazoan databases (Simão, Waterhouse, Ioannidis, Kriventseva & Zdobnov, 2015).

Differential expression analyses and annotation

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Reads from all replicates in each experiment were aligned against the corresponding "reference" transcriptome as per experiment (see Figure 2). Paired reads after trimming were mapped using Bowtie2 v. 2.2.1 (Langmead & Salzberg, 2012) as implemented in Trinity (Grabherr et al., 2011). RSEM v. 1.2.11 (Li & Dewey, 2011) was then run to generate a table with read counts, and unmapped reads were discarded. In the "reference" transcriptomes, transcripts of the same trinity component were treated as different isoforms. We retained information of differential expression of all isoforms detected for a given gene (or component) because they may have different functions. In order to identify common genes and/or isoforms differentially expressed under temperature treatments, the *de novo* assemblies of both experiments, that assigned different transcript names to all isoforms, were blasted against each other using BLASTn.

Differential expression (DE) analyses of the two experiments were performed with the package DESeq2 (Love, Huber & Anders, 2014) in R v 3.2.1 (R Development Core Team 2008). Before performing the analyses, preliminary tests to investigate differences in gene expression between sexes and treatments were performed in DESeq2. No significant differences in response to treatments were observed between males and females (p-adjusted > 0.01), and "sex" was not considered as a variable in further analyses.

For differential gene expression analyses, read counts were first normalized in DESeq2, and then a negative binomial model was fit to accurately estimate differential expression. The significance value for multiple comparisons was adjusted to 0.01 with the function "padj" (Benjamini-Hochberg adjustment) as implemented in DESeq2. Transcripts with significantly different expression values relative to the controls will be hereafter called "DE" transcripts. Component Analyses (PCAs) were performed and plotted with the same package to visualize variation of expression levels among samples and treatments. Visualization of the significant outcomes of isoforms differentially expressed (up- and down-regulated) between treatments of each experiment was obtained with a heatmap performed with the "gplots" package of R (Warnes, Bolker, Bonebakker & Gentleman, 2016).

Using the GO annotation results from the *de novo* assemblies of the two experiments, we

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obtained the GO terms associated to the differentially expressed isoforms, which were then input (together with their associated log_2fold-change) to the REVIGO web server (Supek, Bošnjak, Škunca, & Šmuc, 2011) to obtain summaries of GO terms. Results were graphically represented with the "treemap" R package. Size of the rectangles was adjusted to reflect the log_2fold-change in REVIGO. Differentially expressed isoforms without blast hit, unknown function and/or without annotation for each experiment were further assessed with the InterProScan 5 software (Jones et al., 2014), which predicts protein family membership and the presence of functional domains and sites, at the Superfamily level (De Lima Morais et al., 2011). The InterproScan was run as implemented in the Blast2GO software with default parameters. We finally merged the results of the associated GO terms and those from InterProScan with the purpose of increasing our knowledge of coelomocyte gene functions and GO annotations.

RESULTS

Data filtering and de novo *assembly*

A total of 18 RNA-seq datasets were generated in this study. For *de novo* assembling of each reference (see Figure 2) we used 12 RNA-seq datasets. For quantifying transcriptomic responses, we used 11 datasets per experiment since one sample from treatment T7 and another from T22 were discarded for gene expression analyses because of their low quality (see Figure 1). Datasets have been deposited in Mendeley Data (doi.org/10.17632/5673n552yj.1) and the NCBI (BioProject nº PRJNA642021). The number of trimmed reads used for *de novo* assembly, as per sample replicate and treatment, are detailed in Supplemental Information 1 (Tables S1 and S2). All replicates had over 26 million reads.

The *de novo* assembly "CT+T7", used as a reference for the "Control *vs* T7" experiment, included 141.5 Megabases that rendered 211,650 transcripts (including both genes and their different

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isoforms), and 19.6% of them had blast hit with known proteins of metazoans (see species blast hit distribution in Supplemental Information 1, Figure S2). The reference assembly "CT+T22" for the "Control *vs* T22" experiment included 147.4 Megabases, and rendered 219,655 different transcripts, from which 17.9% had blast hit (see species blast hit distribution in Supplemental Information 1, Figure S2). Both *de novo* assemblies were very comparable (and had 99.5% transcripts in common), presenting relatively high N_{50} values, between 1,102 and 1,114, meaning that over 50% of the transcripts were longer than 1,100 bases. Details of the *de novo* assemblies for the two different experiments are presented in Supplemental Information 1 (Table S1). Both, "CT+T7" and "CT+T22", showed high completeness when compared with BUSCO conserved ortholog databases of eukaryotes and metazoans (see Supplemental Information 1, Table S3). For the reference assemblies, "CT+T7" and "CT+T22", 194 and 4,293 transcripts, respectively, had blast hits against proteins and/ or nucleotides of bacteria and were removed from subsequent analyses. In fact, most differences between the reference assemblies "CT+T7" and "CT+T22" were due to the amount of bacterial transcripts.

General results of differential expression analyses

The differential expression analyses revealed changes in gene expression between controls and temperature treatments in both experiments, "Control *vs* T7" and "Control *vs* T22". Additionally, we detected a remarkable difference in the magnitude of the transcriptomic responses between experiments, which was over six-fold greater in number of DE transcripts in the "Control *vs* T7" experiment, as explained below. We also observed differences in gene expression among different isoforms of the same genes.

In the "Control *vs* T7" experiment, we detected 1,181 DE transcripts between CT and T7, with 720 transcripts up-regulated at T7 (61% of the total DE transcripts) and 461 transcripts down-regulated at T7 (49% of the total DE transcripts) (see Figure 3). A total of 445 transcripts (37.7 % of the total DE transcripts) had blast hit and known function (see Supplemental Information 1, Table S4), including 28 transcripts within the top 50 most significant DE (see Supplemental Information 2).

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Regarding isoforms, over all DE transcripts (potential genes), 176 presented different isoforms (see Supplemental Information 2). Ten genes presented all isoforms DE between CT and T7, whereas the other 166 genes only showed some of their isoforms differentially expressed between treatments.

The number of DE transcripts in the "Control *vs* T22" experiment was much lower than in the "Control *vs* T7" experiment, with only 179 DE transcripts, being 57 transcripts up-regulated (32% of the total DE transcripts) and 122 transcripts down-regulated (68% of the total DE transcripts) in the T22 treatment (see Figure 2). Only 35 transcripts (19.7 % of the total DE transcripts) were assigned annotation and known function (Supplemental Information 1, Table S4), 10 of them within the top 50 most significant DE. Of these 35 transcripts, 27 had different isoforms, and in all cases only one of their isoforms was DE between CT and T22 (see Supplemental Information 2). A complete list of differentially expressed, annotated transcripts for both experiments is presented as Supplemental Information (2), including transcript identification code (id), logarithm of the fold change, adjusted *p*-value with FDR correction obtained from the expression analyses, gene description, number of isoforms found and transcripts with known function within the top 50 most significant DE (* Top 50 DE). Fourteen DE transcripts were common between experiments (see Figure 3) and most of them featured opposite responses between treatments. Only four of these transcripts had annotations; three of them showed opposite responses between the two experiments (*fam-55cc* with $\log_2$fold-change=-6.01 and 5.47, *tripartite motif-containing protein* 3 with $\log_2$fold-change=4.51 and -6.36, and *wsc domain- containing protein 1* with $\log_2$fold-change=-1.19 and 1.84 at "Control *vs* T7" and "Control *vs* T22" experiments, respectively), whereas the *histone-lysine n-methyltransferase prdm 9* was down-regulated in both temperature treatments, T7 and T22, of the two experiments.

Figures 4 and 5 showed, in general, little differentiation between replicates of the same treatment, and large differences in transcript expression between treatments. Only one of the control replicates from the "Control *vs* T22" experiment had a mixed pattern of expression between control and treatment samples (Control replicate 1, see Figure 4), and clustered with the treatment samples on the PCA (see Figure 5). However, this same control sample did not follow the same trend in the other experiment ("Control *vs* T7") and grouped neatly with the other control samples (Figures 4 and 5).

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A total of 84 and three GO terms were found associated to DE genes in the "Control *vs* T7" and "Control *vs* T22" experiments, respectively (Supplemental Information 1, Table S4). The InterProScan could only predict information of protein domains in six uncharacterised transcripts of the "Control *vs* T22" experiment. In Figures 6 and 7 the up- and down-regulated GO categories associated to DE transcripts from the two experiments are depicted. These GO terms were not equally represented among categories between up- and down-regulated DE transcripts, or between experiments. For the experiment "Control *vs* T7" the most important up-regulated GO term categories (Figure 6) were: for Biological Process (BP),"tyrosine metabolism" (including "positive regulation of apoptotic process"), "peptidyl-tyrosine dephosphorylation", "protein folding" and "ATP hydrolysis coupled proton transport". For Cellular Component (CC), "proton-transporting V-type ATPase-V0 domain" and "sarcoplasmic reticulum"). For Molecular Function (MF),"GTP binding", "protein tyrosine phosphatase activity", "Protein tyrosine phosphatase activity", "sulfo-transferase activity", "hydrogen ion transmembrane transporter activity" and "lipid binding" (among others). Among the most important down-regulated GO categories (figure 6) we found: for BP, "neurotransmitter transport", "intracellular signal transduction" and "protein O-linked glycosylation". For CC, "nuclear origin of replication recognition complex", "cell", "intracellular" and "integral component of membrane". For MF, "protein-N-acetylglucosaminyltransferase activity", "sequence- specific DNA binding", NAD-dependent histone deacetylase activity" and "zinc ion binding" (among others). For the experiment "Control *vs* T22" only GO information for down regulated transcripts could be obtained and, among them, the most important DE categories were "notch signalling pathway", "multicellular organismal development" for BP, "integral component of membrane", "membrane", and "SAGA-type complex" for CC, and "calcium ion" and "protein binding" for MF.

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

At 7˚C, the expression of genes encoding different heat shock proteins was up-regulated,

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including transcripts for the Heat Shock family proteins: an inducible *Hsp70* (Supplemental Information 2, Figure S3), and *Hsp71*, *Hsp90* and the *Dnaj homolog subfamily c member 21(DNAJC21)*, which encodes a member of the DNAJ heat shock protein 40 family (*Hsp40*), which acts as a co-chaperone of *Hsp70* (Supplemental Information 2, Figure S3). See Supplemental Information 2 for *Hsp40* transcripts and foldchanges: c256938_g1_i3, log_2fold-change= 2.98; c260821_g2_i1, log_2fold-change= 1.35; c260821_g1_i2, log_2fold-change= 3.05; c264479_g1_i1, log_2fold-change= 7.35; c249691_g1_i1, log_2fold-change= 1.91; c271252_g1_i1, log_2fold-change= 3.69. In addition, the receptor of stress *Wsc domain-containing protein 1* was found down-regulated at 7˚C and up-regulated at 22˚C (Supplemental Information 2: c266025_g2_i1, log_2fold-change= -1.19; and c265343_g1_i1, log_2fold-change= 1.84, respectively).

Several transcripts from the apoptotic gene complements were differentially expressed between controls and T7. They included the *Bcl2* (up-regulated in T7, Supplemental Information 2: c263429_g1_i1, log_2fold-change= 2.17; and c271119_g2_i1, log_2fold-change= 1.73), *sequestosome 1* (up-regulated in T7, Supplemental Information 2: c257995_g1_i1, log_2fold-change= 3.72) and *fas-associating death domain-containing protein* and *death ligand signal enhancer* (down- and up-regulated in T7, respectively; Supplemental Information 2: c268119_g1_i3, log2foldchange= -1.48 and c270362_g1_i1, log_2fold-change= 1.99). In T22, we found upregulation of *immediate early response 3-interacting protein 1-like* (Supplemental Information 2: c276658_g1_i2, log_2fold-change= 1.55).

At 7˚C, there was an up-regulation of genes involved in the innate immune response identified as *echinoidin*, *senescence associated-gene* and *Tripartite motif-containing protein 3* (TRIM) (Supplemental Information 2: c258741_g1_i1, log_2fold-change= 5.35; c150071_g1_i1, log_2fold-change= 6.02; c273778_g2_i1, log_2fold-change= 2.90). In addition, the genes *interleukin-17* and cytohesin-like were also upregulated in T7 (Supplemental Information 2: c239836_g1_i1, log_2fold-change= 6.22; and c263807_g1_i1, log_2fold-change=1.77, respectively).

DISCUSSION

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484

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

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

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

Decreasing temperatures elicited the activation of genes related to metabolic changes, pro- and anti-apoptotic mechanisms, and immune responses in coelomocytes of *A. lixula*. Among the up-regulated genes related to the stress response at 7˚C, we detected the *Hsp71*, *Hsp90*, an inducible *Hsp70*, and *Hsp40*; with the last one being a co-chaperone of the *Hsp70*. The protein *Hsp40s* stimulate the ATPase activity of *Hsp70s* and targets unfolded proteins to *Hsp70s* (Ngosuwan, Wang, Fung & Chirico, 2003). In general, these HSP chaperones are involved in the strong and mild thermal stress responses, including protein folding reactions to avoid protein denaturation of adults, early development stages and eggs of sea urchins (e.g. Matranga et al., 2000, 2002; Runcie et al., 2012; González, Gaitán-Espitia, Font, Cárdenas & González-Aravena, 2016). Their presence might be involved in the wide thermal distribution of some particular marine species (see Zhu et al., 2016, and references herein), and the HSP family seems to be a mechanism to cope with the stress associated with cold in *A. lixula* (e.g. NW Mediterranean). On the other hand, no up-regulation of genes encoding HSPs was detected at 22˚C relative to the control condition in *A. lixula*.

Under conditions of thermal stress, protein refolding by HSPs may not be efficient enough, and misfolded protein degradation can be necessary to restore cell homeostasis (Mosser et al., 2000). Therefore, other mechanisms such as proteolysis to eliminate disfunctional proteins via the *Ubiquitin* proteosome pathway and, finally, apoptosis to eliminate damaged cells, can be activated (Somero, 2010; Logan & Somero, 2011; Zhu et al., 2016). We only detected signs of *Ubiquitin* proteosome pathway activation (up-regulation of some genes involved in this pathway) in the 7˚C treatment, with

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the up-regulation of the gene *sequestosome 1* (Supplemental Information 2), which is an autophagosome cargo that detects proteins for autophagy and has been previously identified in echinoderms (Bitto et al. 2014), and the *e3 ubiquitin-protein ligase*, which targets damaged proteins for transport and degradation by the proteasome (Ardley & Robinson, 2005).

In addition, we observed differential expression of several apoptosis-associated genes in both treatments, 7˚C and 22˚C. Several studies demonstrated that sea urchins hold a complex apoptotic system (Agnello & Roccheri 2010; Lesser, 2012). We found transcriptional changes at 7˚C in apoptosis suppressor genes such as the *Bcl2* (up-regulated, Supplemental Information 2), widely distributed in different marine invertebrates (see Lesser, 2012), and in genes containing death domains (down-regulated: *fas-associating death domain-containing protein* and *death ligand signal enhancer,* Supplemental Information 2) that induce cell apoptosis through the regulation of caspase activation (Agnello & Roccheri 2010; Zhu et al., 2016). These findings suggest the activation of some particular pathways to control the programmed cell death at low temperatures. The up-regulation at 22˚C of the gene *immediate early response 3-interacting protein 1-like* (Supplemental Information 2), which is a molecule involved in protein transport between the Sarcoplasmic reticulum and Golgi apparatus and that mediates apoptosis in human cells (https://www.uniprot.org), suggests that apoptosis is also occurring as a response to increased experimental temperatures.

Additionally, a *Serine threonine- protein kinase pim3*, an enzyme involved in the regulation of cell transport and survival, which prevents apoptosis by inducing the release of the anti-apoptotic *Bcl2* mentioned before (Cross et al., 2000) was also overexpressed at 7˚C, whereas a *Serine threonine-protein phosphatase 6,* with opposite function to the kinase enzyme (Cross et al., 2000), was down-regulated at 22˚C. Another interesting finding is the opposite pattern of gene expression found between experiments for the *Wsc domain-containing protein 1* (down-regulated at 7˚C and up-regulated at 22˚C) (Supplemental Information 2). Different members of the *Wsc* family are identified as putative receptors of stress and required for the heat shock response and the maintenance of cell wall integrity in yeasts (Lodder, Lee & Ballester, 1999). The *Wsc* members are upstream regulators of other serine-threonine kinases, the protein kinase C1 (PKC1) and mitogen-activated protein kinase

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

Previous experiments on echinoderms demonstrated the effect of thermal stress on the immune capacity of coelomocytes; this effect is greater at higher rather than lower temperatures in the sea cucumber *Apostichopus japonicas* (Wang, Yang, Gao & Liu, 2008). However, in *A. lixula*, it was the lowest temperature the one that triggered a higher immune response in terms of gene expression. The *echinoidin*, *senescence associated-gene*, *cytohesin-like* and *tripartite motif-containing protein 3* (TRIM) (Supplemental Information 2) involved in the infection response and/or pathogen-recognition process against bacteria, fungi and viruses (Smith et al., 2006; Ozato, Shing & Chang, 2008) were up-regulated at 7ºC. In addition, the gene *interleukin-17* (Supplemental Information 2) which is a cytokine inducing and mediates proinflammatory responses in metazoans and stimulates phagocytosis in echinoderms (Beck et al. 1993), was also up-regulated at 7˚C. None of these immune genes were, however, up-regulated (or, when detected, were down-regulated) at the highest experimental temperature (e.g. TRIM), suggesting no immune response at 22˚C.

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

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different cell lineages, it was shown that hyperthermia reduced *Notch1* expression and apoptosis in some cell lineages, whereas an opposite pattern was obtained in other cell lineages (Basile et al., 2007). Although the effect of the Notch down-regulation at high temperatures in coelomocytes is not completely clear, it suggests the existence of an alternative pathway of apoptosis under thermal stress.

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

Among the down- regulated GO terms at the 7˚C treatment we found "Neurotransmitter

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

Another remarkable result from our work is the large number of gene isoforms found in *A. lixula* transcriptome. Different gene isoforms are mostly generated by alternative splicing, whose function is to increase the diversity of mRNA expressed by the genome (e.g. Stamm et al., 2005; Kelemen et al., 2013). It has been demonstrated that alternative splicing can promote from neutral or subtle effects on transcripts, and finally proteins functioning, to drastic physiological changes (see a review in Kelemen et al., 2013). Therefore, the presence of different gene isoforms is relevant when

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studying gene expression and physiological pathways. Although our objective was not to analyse differential splicing in *A. lixula*, our results show different levels of expression of some gene isoforms under thermal treatments.

In summary, and despite the limited proportion of annotated transcripts obtained, our results based on RNA-seq analyses of the whole transcriptome of coelomocytes in *A. lixula* show that this NW Mediterranean population in the coldest part of its distribution range (Wangensteen et al. 2012; Pérez-Portela et al. 2019), displays strong gene expression changes in response to the cold treatment, with activation of many genes whose functions could be related to stress responses in the form of chaperone production, apoptosis regulation, ATP-associated genes, enhancement of the immune system and redox processes, and down-regulation of gene pathways related to protein biosynthesis and DNA replication. Nevertheless, contrary to that found in other studies (e.g. Gleason & Burton, 2015; Zhu et al., 2016) no activation of genes encoding antioxidant enzymes was detected in our experiments. As we initially expected, a markedly lower response is found in the warm treatment, with no activation or deactivation of the previously mentioned pathways, with the exception of the apoptosis regulation. Although some caution is needed, as we have characterized transcriptional changes and not protein levels, the differential patterns found in these genes strongly indicated that sea urchins are more stressed under lowered experimental temperatures.

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

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the species inhabits. Additionally, considering different reproductive seasons would be also interesting since organisms may display different thermal sensitivity depending on their gonad maturation stage, and may experience changes in energy allocation. Increasing gene annotation quantity and quality for this species is also desirable to obtain meaningful physiological conclusions in further studies.

It has been suggested that the tropicalization of NW Mediterranean can lead to a shift in dominance between the temperate common sea urchin *Paracentrotus lividus*, which will suffer from warming temperatures, and the thermophilous black sea urchin *A. lixula* (Gianguzza et al., 2011, Wangensteen et al., 2013a,b, Carreras et al., 2020). Such a shift can have drastic ecological impacts, as both species are conspicuous engineer species shaping benthic communities (Bulleri et al., 1999, Bonaviri et al., 2011). Specific biological and genomic studies are needed to understand the adaptive capabilities of *A. lixula* to ongoing warming, but our results add to the available evidence that colder rather than warmer temperatures may be a limiting factor for *A. lixula*. The absence of clear signs of stress at warm temperatures in adults of *A. lixula,* together with information on larvae development and gonad maturation (Wangensteen et al., 2013a and 2013b), support the hypothesis of a positive effect of winter warming on the species' reproduction output and larval survival. The ongoing expansion of the species across the littoral coast of the Mediterranean, with the concomitant impacts of its grazing activity on littoral communities, may be exacerbated in the near future by rising winter temperatures in the NW Mediterranean.

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DATA ACCESSIBILITY

The *De novo* assemblies, RSEM, annotation and DEseq files are available at Mendeley Data doi.org/10.17632/5673n552yj.1. Raw reads are available at the Sequence Read Archive under the BioProject number PRJNA642021 and BioSamples SAMN15375803-SAMN15375820.

AUTHOR CONTRIBUTIONS

All authors contributed to the design of this study and were involved in the aquarium experiments. RPP, AR, XT and OSW analysed the data. RPP wrote the first draft of the manuscript and created figures and tables. XT, AR and CP contributed to improve the first draft, and all authors revised the final version of the manuscript.

FIGURE LEGENDS

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Figure 1. Sampling and experiments in *Arbacia lixula*. A- Samples analysed for gene expression: Comparison between Control condition (CT at 13°C) and temperature at 7°C (CONTROL *vs* T7), and Control and 22°C (CONTROL *vs* T22). Red crosses indicated replicates lost during the development of the experiments, and B- Map of the sampling area of *A. lixu*la.

Figure 2. Pipeline followed in this study. The most important experimental steps and analyses are represented.

Figure 3. Number of differentially expressed (DE) transcripts between treatments and experiments. A- Comparison of number and percentage of up- and down- regulated transcripts between treatments at each experiment, and B- Venn diagram representing the number of DE transcripts per experiment and those (14) in common between experiments.

Figure 4. Heatmaps based on differentially expressed transcripts (DE) from pairwise comparisons of treatments within experiments. A- Control condition versus Temperature 7°C, and B- Control condition versus Temperature 22°C. Different colours indicate relative expression levels. Similarity in gene expression patterns among replicates (individuals) is represented by clustering on the top of the heatmaps.

Figure 5. Principal Component Analyses (PCAs) plots for the two different experiments including all replicates per treatment. A- "Control versus Temperature 7°C", and B- "Control versus Temperature 22°C"

Figure 6. Gene Ontology treemaps for annotated differentially expressed genes in Control versus Temperature 7°C. DOWN- and UP- regulated categories at 7°C are presented as separated figures for Biological Processes and Cellular components. Molecular functions are presented in Supplemental Information 1 (Figure S4). The size of the rectangles reflects the log_2fold-change associated to the differentially regulated categories.
[**1**= multicellular organism development; **2**= positive regulation of GTPase activity; **3**= regulation of ARF protein signal transduction; **4** single−organism cellular process**; 5**= DNA replication; **6**= polyamine biosynthetic process; **7**= histone H3 deacetylation; **8**= DNA−templated transcription initiation; **9**= microtubule−based movement; **10**= cation transport; **11**= trans-membrane transport; **12**= ER to Golgi vesicle-mediated transport; **13**= peroxisome fission; **14**= mitochondrion organization; **15**= ammonium ion metabolism; **16**= metabolism; **17**= nucleus; **18**= transcription factor TFIID complex; **19**= integral component of peroxisomal membrane; **20**= tubulin complex; **21**= cytoplasm; **22**= pantothenate biosynthetic process; **23**= microtubule−based process; **24**= RNA secondary structure unwinding; **25**= oxidation-reduction process; **26**= vesicle docking involved in exocytosis; **27**= protein transport; **28**= metabolism; **29**= methylation; **30**= single organism process; **31**= protein polymerization; **32**= nucleosome; **33**= microtubule; **34**= transcription factor complex; **35**= protein phosphatase type 2A complex; **36**= cytoplasm; **37**= cytoplasmic vesicle; **38**= intracellular; **39**= integral component of membrane; **40**= membrane]

Figure 7. Gene Ontology treemaps for annotated differentially expressed genes in Control versus Temperature 22°C. Only the function of DOWN- regulated genes at 22°C was obtained for Biological Processes, Cellular components, and Molecular functions. The size of the rectangles reflects the log_2fold-change associated to the differentially regulated categories.

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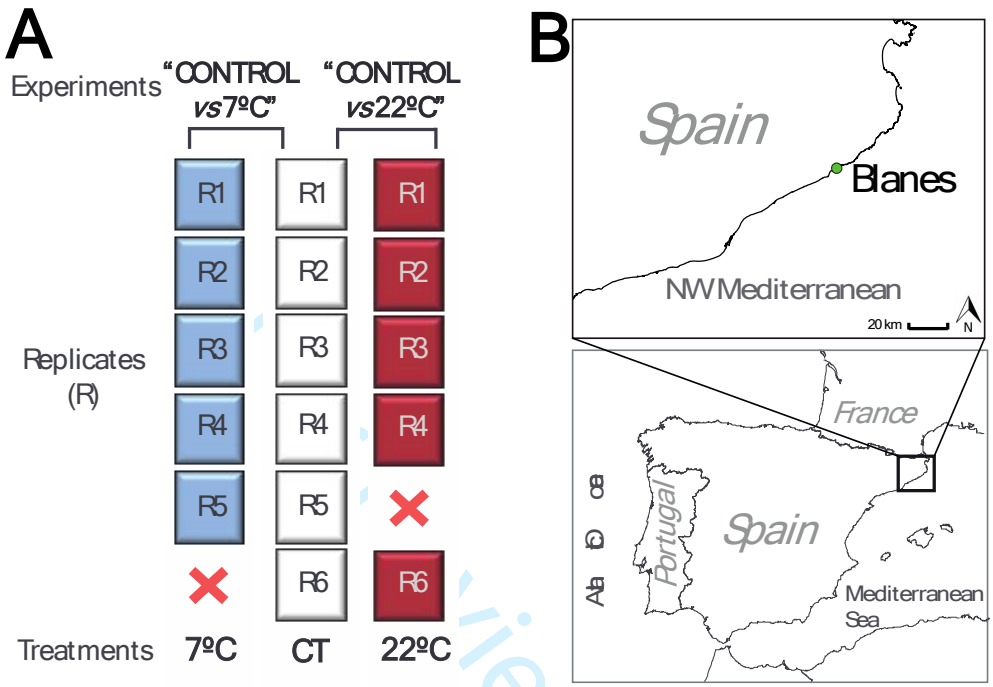

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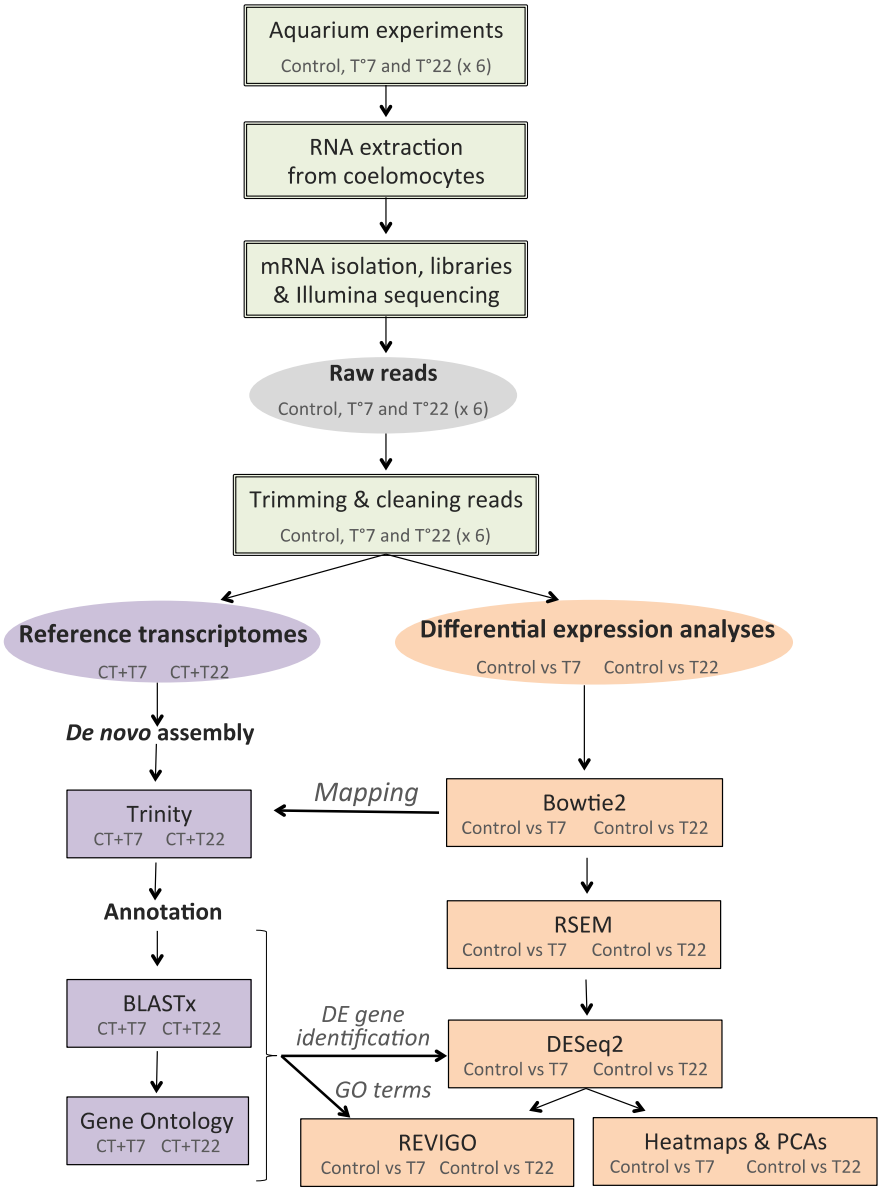

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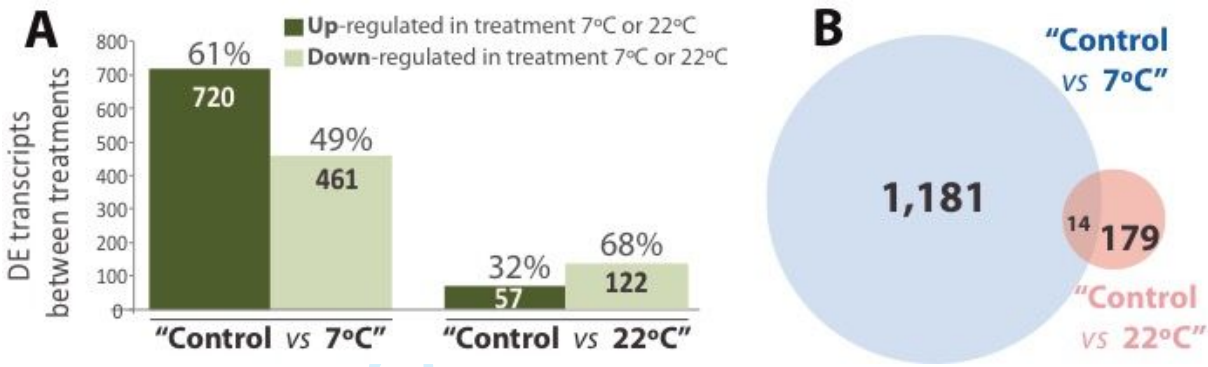

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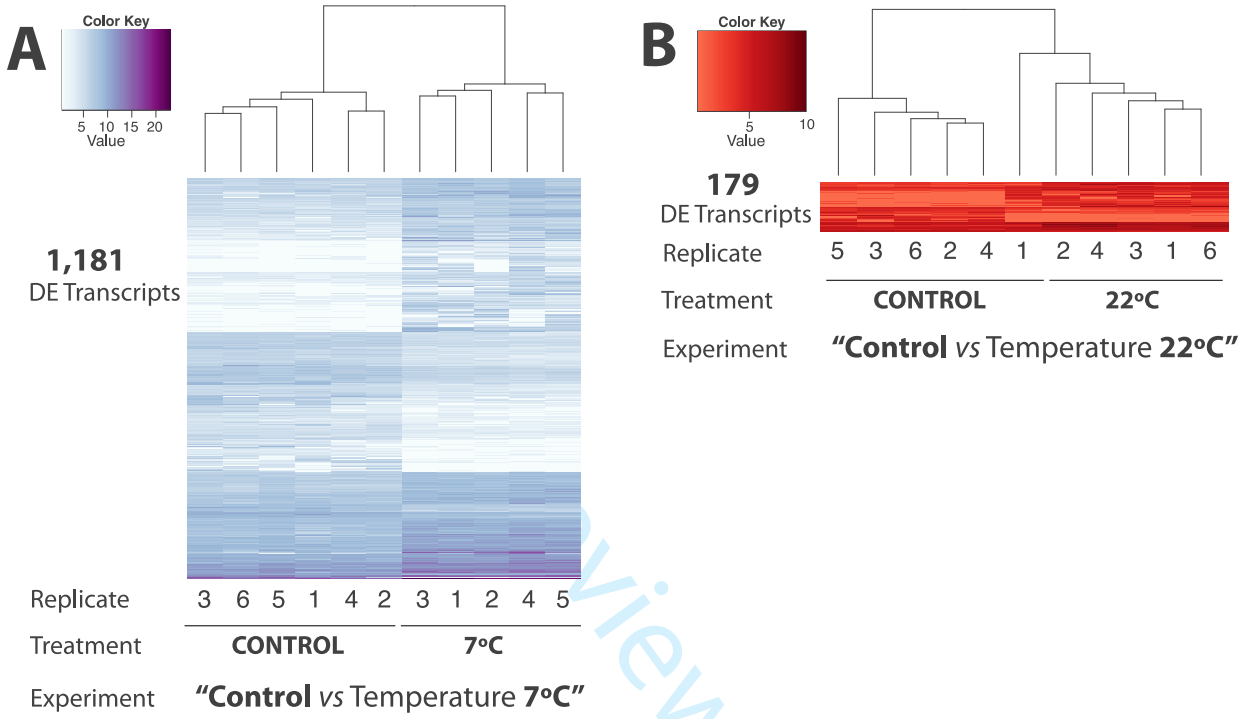

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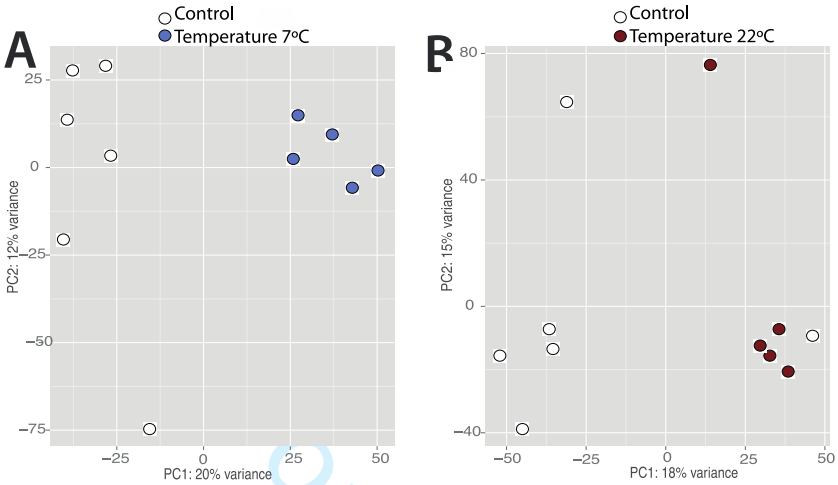

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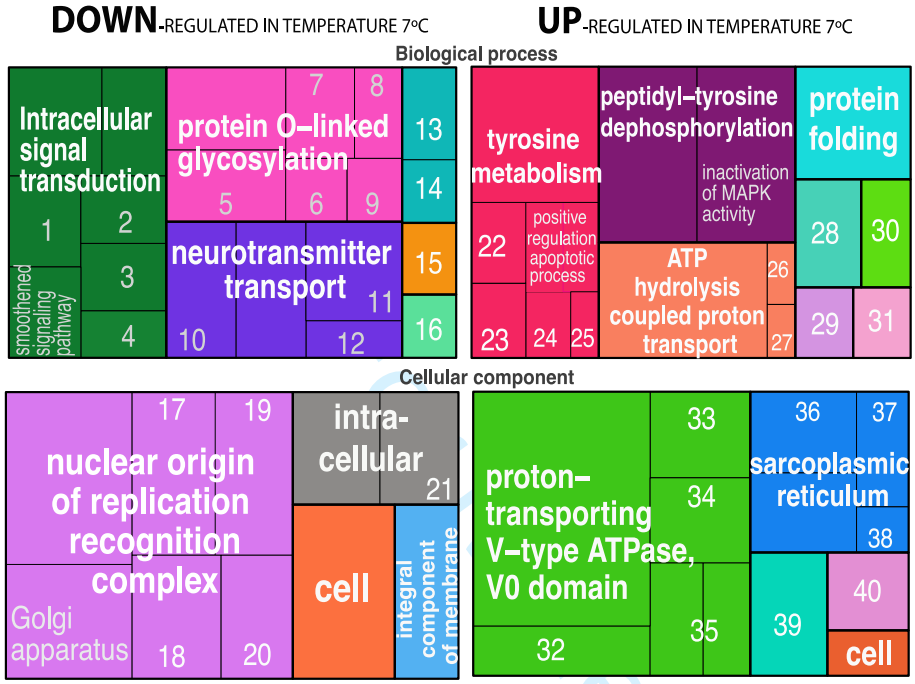

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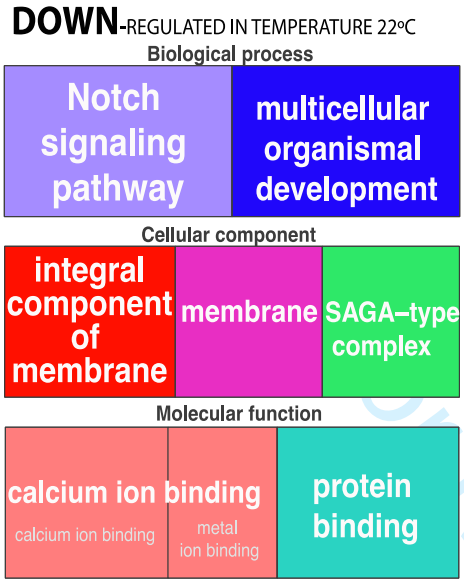

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