Warm Temperatures, Cool Sponges: The Effect of Increased Temperatures on the Antarctic Sponge *Isodictya* sp.

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Abstract

Although the cellular and molecular responses to exposure to relatively high temperatures (acute thermal stress or heat shock) have been studied previously, only sparse empirical evidence of how it affects cold-water species is available. As climate change becomes more pronounced in areas such as the Western Antarctic Peninsula, it has become crucial to understand the capacity of these species to respond to thermal stress.

Here we use the Antarctic sponge *Isodictya* sp. to investigate how sessile organisms (particularly Porifera) can adjust to acute short-term heat stress, by exposing this species to 3 and 5 °C for 4 hours, corresponding to predicted temperatures under high-end 2080 IPCC-SRES scenarios. Assembling a *de novo* reference transcriptome (90,188 contigs, >93.7% metazoan BUSCO genes) we have begun to discern the molecular componentry employed by *Isodictya* to adjust to environmental insult.

Our initial analyses suggest that TGF-β, ubiquitin and hedgehog cascades are involved, alongside other genes. However, the degree and type of response changed little from 3 to 5 °C, suggesting that even moderate rises in temperature could cause stress at the limits of this organism's capacity. Given the importance of sponges to Antarctic ecosystems, our findings are vital for discerning the consequences of increases in Antarctic ocean temperature on these and other species.

Key Words:

Porifera; Thermal stress; Climate change; Transcriptome; Western Antarctic Peninsula

Background

Sponges are vital components of a variety of ecosystems in terms of abundance and ecosystem services[1,2]. This is especially true in the Antarctic, where they are among the most common benthic species, providing habitat and food for a wide range of organisms[3,4,5,6]. Sponges, like most marine species, will be broadly, and perhaps adversely, affected by climate change, but at present studies into their capacity to cope with increases in temperature are limited in scope and number[7,8].

The Western Antarctic Peninsula (WAP) is one of the areas of the planet which has experienced some of the most significant changes in air and water temperature[9,10]. Current estimates of climate change in the Antarctic suggest that the mean temperature of seawater could rise by up to 5 °C by 2080 (IPCC 2001, Table 16.1) although more recent IPCC reports put the best estimate for this at about + 0.6°C (Representative Concentration Pathway 2.6) to 2.0°C (Representative Concentration Pathway 8.5) (IPCC 2014)[11]. This is expected to have major implications for Antarctic organisms as they have evolved in a very stable and cold environment, being highly sensitive to environmental variation[12,13]. For this reason the projected changes in water temperature constitutes a major threat to ecosystem function in these waters. Such changes would modify the prevalence of key functional species, thus affecting associated ecosystem processes[14]. However, we are only beginning to understand the resilience of many species found in the Antarctic to rising temperature conditions [12,15,16,17], and the point to which they will be affected by any changes in mean temperature. The deleterious effects of temperature exposure in sponges from other latitudes include bleaching of symbionts, tissue necrosis and death[18]. It is vital to gain this information in Antarctic sponge species, so that policy decisions can be made with a full understanding of the likely impacts of these changes.

Some sponge species have been suggested to be relatively robust to moderate changes in temperature. Caribbean and Brazilian coral-reef sponges have been investigated and found

77 to survive fluctuations in ambient temperature[19,20]. A recent study, experimentally demonstrated
78 that the boreal deep-sea sponge *Geodia barretti* is able to cope with temperature rises with few
79 ill effects[21]. Other sponge species are however not so resilient [18,22,23], and both reproduction[24]
80 and filtration[25] in sponges has been shown to be affected by increases in temperature. In some
81 cases, this has led to widespread mortality [22,26].

82 We have little current understanding as to what makes some species able to cope with
83 broad temperature ranges, and it is possible that Antarctic-dwelling species may be particularly
84 vulnerable due to their specialisation for extremely cold, relatively stable temperature
85 conditions[27]. Their molecular components in particular may have altered over evolutionary time,
86 as has been observed previously in a variety of species [17,28,29], and warmer temperatures could
87 prove deleterious. The sponge *Isodictya* sp. (Fig 1A) is no exception to this. While this sponge is
88 commonly observed around the WAP and generally lives in waters with temperatures between -
89 1.8 to 2 °C[30], it is rarely found in warmer areas. Increasingly this sponge is exposed to warmer
90 summer temperatures across its natural range, with shallow-water temperatures reaching 3 °C
91 in some places around the WAP[31].

92 Illumina-based sequencing can reveal both subtle and broad changes in expression[32].
93 The decreasing cost of sequencing and steady increase in affordable computational power has
94 made such approaches increasingly viable, even for small lab teams and consortia[33]. These
95 studies have the additional benefit of also providing a suite of information on the genetic
96 componentry possessed by the organism in question, which is particularly useful for non-model
97 species. While genomic-level datasets within the Porifera remain depauperate and limit our
98 understanding of adaptation to particular conditions, a number of investigations into the
99 transcriptomic complements of a variety of sponges have now been successfully performed
100 [34,35,36]. The transcriptional changes in response to temperature shifts that occur within sponges
101 themselves[23,37] and changes in their bacterial symbionts[18,38,39,40.41] have also only recently begun
102 to be unveiled. Some changes in gene expression revealed by these studies, such as those in

103 HSP70 expression[37,42] are easily explained with reference to known metazoan responses to
104 temperature fluctuation. Similarly, responses such as the up-regulation of genes involved in
105 signal transduction, tissue morphogenesis, cell signalling and antioxidant activity are all of clear
106 utility to tissues as they adapt to thermal stresses. What has yet to be tested thoroughly is
107 whether these changes are mirrored in extremophilic species. It is possible that gene
108 complements adapted to extremes may be less able to adapt to changes, particularly at rapid
109 time scales.

110 Our study demonstrates that some extremophile species such as *Isodictya* sp. may be
111 able to respond to acute short term stress, but may not have the capacity to adjust further to
112 continued rises in temperature. Using a small number of replicates and two "heat shock"
113 regimes, we have been able to use these findings to investigate the specific pathways used by
114 these organisms, and contrast these with species adapted to warmer temperature regimes. The
115 sponge studied here, *Isodictya* sp. is one of the most common species in shallow-water rocky
116 reefs (<25 m depth) around Doumer Island, Palmer Archipelago, WAP (Fig 1B). This knowledge
117 will be vital for understanding the impact of temperature rises on Antarctic species, but also for
118 discrete, targeted investigation of the role of specific pathways in temperature adaptation in the
119 future. In this manuscript we present the first transcriptomic analysis of the effect of acute
120 warming on a cold-adapted sponge.

121

122 **Methods**

123

124 *Animal Collection and Heat Treatment*

125 Adult samples of six individuals of the Antarctic sponge *Isodictya* sp. were collected by
126 SCUBA diving at 10 m depth in Cape Kemp, off Yelcho Research Station, Doumer Island, WAP
127 (64°51′S 63°35′W, Fig. 1B) in January 2017. Sponges were then transferred to the laboratory at
128 Yelcho Station, where they were maintained in 140 L fiberglass tanks with unfiltered, flowing

129 seawater (approximately 0.5 °C, pumped directly from the sea floor near to collection site (5 -10
130 m depth)) for a week to allow acclimation to laboratory conditions. Tanks were covered with two
131 layers of 1 mm shade cloth (fiberglass 50% neutral density screen) to represent light levels
132 occurring *in situ*. Relative light intensity levels and temperature in the experimental tanks were
133 measured (1 measurement every 15 minutes) during the duration of experiment with a HOBO
134 Pendant® temperature/light data logger (Onset, USA). Sponges were then placed into one of
135 three possible treatment conditions - a control tank, where samples were maintained in
136 seawater pumped directly from the sea floor at approximately 0.5 °C, or one of two possible
137 treatment temperatures, 3 and 5 °C, with water warmed in a header tank before pumping into
138 the treatment area, and subsequently maintained at a set temperature using SOBO Aquarium
139 heaters (500 watt). These temperatures were chosen in line with empirical evidence of warming
140 in the WAP[31] and to correspond to the higher possible estimates of warming in the Antarctic
141 (IPCC, 2001 report). Sponge samples were placed in individual treatment tanks, for a total of 3
142 treatments and two replicates (2x control, 2x 3 °C, 2x 5 °C) although one control sample was
143 not used for analysis as described in Results and Discussion.
144
145 *RNA Extraction and Sequencing*
146 Sponges were taken from tanks after 4 hours of exposure to the control/treatment
147 conditions, and a single fragment of each sample, approx. 1 cm^3, was taken immediately and
148 placed in RNAlater (Qiagen) and stored at -20 °C on site, before shipment on dry ice (with an
149 unbroken cold chain) from Yelcho via Escudero Station to a molecular laboratory at the
150 Laboratorio de Biorrecursos at INACH, Punta Arenas. RNA was extracted from sponge samples
151 using E.Z.N.A Total RNA kit II (Omega Bio-Tek Inc.) according to the manufacturer's protocol
152 with a previous step of homogenization with liquid nitrogen. RNA quality and quantity was
153 determined on a 2100 Bioanalyser (Agilent Technologies). Quality control obtained an average
154 RIN value of 8.2. The sole sample with a RIN value inferior to 7 (a control sample, with RIN 6.2)

155 was not included in this work. 1 µg of RNA from each sample was used to produce RNA
156 libraries for sequencing, with TruSeq Stranded mRNA kit (Illumina), 100bp nominal intra-read
157 fragment size, 15 rounds of amplification and adaptors AGTTCC, ATGTCA, CCGTCC,
158 GTCCGC and CGATGT.

159 Libraries were sequenced on a Hiseq2500 sequencer by the Macrogen provider, using
160 approximately one half of a run in total. The sequencing provider performed initial assessment
161 of read quality and de-multiplexing of reads according to their procedures, and provided us with
162 paired-end reads for download from an external server, with no unpaired orphan reads retained
163 by this process.

164

165 *Quality Control and Assembly*

166 We confirmed the removal of adapter sequences and overall sequence quality using the
167 FastQC program[43]. Low-quality regions of reads were trimmed using Trimmomatic 0.33[44] with
168 the following settings: ILLUMINACLIP:../Adaptors.fa:2:30:10 LEADING:3 TRAILING:3
169 SLIDINGWINDOW:4:20 MINLEN:30 where the Adaptors.fa file consisted of the appropriate
170 indexes for the libraries in question. The resulting trimmed reads were then compressed using
171 gzip and re-analysed with FastQC. Seqtk fqchk[45] was used to determine average sequence
172 quality scores.

173 Trimmed reads from all 5 samples were assembled into a reference transcriptome using
174 Trinity version 2013_08_14[46], with two non-standard settings: a minimum contig length of 200
175 bp and *in silico* read normalisation. DeconSeq standalone version 0.4.3[47] was used to remove
176 contamination, with settings -i 50 -c 50, using pre-prepared bacterial, archean, hsref and viral
177 databases (2,206, 155, 1 and 3,761 complete genomes respectively), resulting in our final
178 reference assembly. This is available from the DOI and URL below as Supplementary File 1.

179

180 *Phylogenetic Analysis*

181 28S and HSP70 sequences were extracted from our assemblies using BLASTN[48] on a
182 local server, using sequences of known orthology from Genbank as search queries. These were
183 aligned to previously published sequences downloaded from the NCBI database using MAFFT
184 [49]. HSP70 gene alignments were cured using Gblocks[50], although this was not necessary for the
185 well-conserved 28S sequence. Phylogenetic analysis was run in RAxML GUI[51] using ML +
186 Rapid Bootstrap, the GTR model (28S)/LG+I+G model (HSP70) and 1,000 bootstrap replicates.
187 Phylogenetic and molecular evolutionary analyses of AIF genes were conducted using MEGA
188 version 7.0[52]. The tree was inferred using the Neighbor-Joining method, based on the alignment
189 of the sequences using ClustalW 2.0[53] (alignment was improved using the Seaview software
190 4.6.5[54]). Resultant tree topologies were evaluated by bootstrap analyses based on 1000
191 replicates.
192
193 *Transcriptome analysis and annotation*
194 To assess the content of the transcriptome BUSCO v1.1b1[55] was run against the
195 reference transcriptome, using the eukaryotic and metazoan Basic Universal Single Copy
196 Orthologue (BUSCO) gene lists to estimate completeness. Annotation of contigs was performed
197 by translating the longest ORF for each contig using the getORF.py python script, taking the
198 longest ORF, then using standalone BLASTP[48] (cutoff *E* value 0.000001) to search against the
199 refseq protein database. This was supplemented using Blast2GO Pro[56], where full InterPro
200 scanning, mapping, annotation (including ANNEX) and enzyme code mapping was performed,
201 the complete annotations for which are available as Supplementary File 2.
202 The KAAS-KEGG automatic annotation server was used to gain an understanding of the
203 recovery of complete pathways in our transcriptome. These were generated using the online
204 tool[57] rather than as integrated into Blast2GO, due to the increased functionality of the
205 standalone server. The bi-directional best hit method was used to identify and annotate the
206 longest orfs from our contigs, with the protein sequences generated earlier used as the basis for

207 these comparisons against a range of eukaryotic species. The maps generated by this were
208 compared directly against extant ones previously generated for the sponge *Amphimedon*
209 *queenslandica,* and the complete annotations are available as Supplementary File 3.
210
211 *Differential Expression Analysis and Over-representation Analysis*
212 Comparative analyses of gene expression were performed using RSEM[58] as packaged
213 within the Trinity[46] module, using Bowtie2[59] for alignment. The three treatments (control, 3 and 5
214 °C) had their replicates (1, 2 and 2 respectively) cross-sample normalised according to Trimmed
215 Mean of M-values. The three treatments were then cross-compared using edgeR[60] within the
216 Trinity[46] perl wrapper script, according to the "classic" pairwise model with a p-value cut off for
217 FDR of 0.0001, a min abs(log2(a/b)) change of 2 (i.e. 4 fold change) and a dispersion setting of
218 0.1. We excluded any differentially expressed contigs where transcription was only detected in a
219 single sample of any of the five used in this analysis, prior to clustering, to avoid spurious results
220 caused by transient expression or contamination of single samples. The p-value cutoff and min
221 abs change values are stringent, to exclude potentially artifactual changes from our analysis,
222 given the small number of replicates. The remaining differentially-expressed gene lists were
223 then targeted for further analysis. These results are provided as Supplementary File 4.
224 GO over-representation analysis was performed in Blast2GO Pro[56] using Fisher's Exact
225 Test, with FDR control for multiple testing, with a p-value cutoff of 0.05[61]. The test sets were the
226 annotated results for the sequences present in each of the over-represented sets derived from
227 the differential expression analysis, analysed in turn, with the reference set the full list of
228 annotated contigs, minus those in the test set.
229
230 **Results and Discussion**
231
232 *Sequencing and Read Cleaning*

233 While 5 individual samples are noted here (a single control, and two replicates for both
234 the 3 °C and 5 °C treatments) another control sample was also initially taken and sequencing
235 performed as described above. Despite being morphologically identical, our initial assays of the
236 sequences from that sample, and particularly the sequence of common molecular markers used
237 in phylogenetic comparison, including *28S rRNA* and *cytochrome c oxidase 1* (*CO1*) markers,
238 revealed that it is in fact a cryptic related species, which will be the subject of description in the
239 future (see Fig. 1C). We have not included this sample in any of the analyses in this work.

240 Basic sequencing metrics can be seen in Table 1, alongside those after read cleaning.
241 Our initial FastQC analysis revealed the presence of low quality nucleotide sequence in the
242 second file of many pairs. Cleaning was therefore stringent, and resulted in markedly fewer, but
243 much better average quality, reads for all samples, which were then used for assembly of the
244 reference transcriptome and for differential expression analysis. GC% (which can be a crude
245 proxy for contamination or changes in expression) was even through all our samples, between
246 44 and 46%, and changed little with cleaning. A small number of over-represented sequences
247 were also initially observed in our reads, as is commonly observed in Illumina-based
248 transcriptomic analysis due to known biases in hexamer binding[62]. The difference in average
249 quality after cleaning was modest, with an average difference of around 2 in Phred score. Our
250 original reads have been uploaded to the NCBI SRA with accession number PRJNA415418.

251

252 *Assembly and Completeness*

253 Reads from all 5 samples were used to construct a reference assembly, for use in
254 further analysis (Supplementary File 1). We checked this for potential contamination using
255 Deconseq, comparing our data to known bacterial, viral, 'archaea' (*sensu* [63], we are aware of
256 archaean paraphyly) and human genomic sequences. With very permissive settings for
257 recognition as foreign sequence (minimum thresholds for removal, 50% similarity across at least
258 50% of the contig length) 797 contigs were tagged as potential contamination. These were

259 removed from our dataset before further analysis. It should be noted, however, that particularly
260 novel bacterial sequences not yet represented in the *nr* database may still be present in our
261 data, despite the use of polyA sequencing, as these could not be represented in our removal
262 database and therefore will not have been removed. While beyond the scope of this manuscript,
263 changes in symbiont content may be vital for long-term adaptation to change.

264 Statistics related to our cleaned reference assembly can be seen in Table 2. A total of
265 90,188 contigs are present, and Trinity, a splice aware assembler, has automatically assigned
266 70,844 as independent "genes". A small amount of heterozygosity or potential splice variation
267 has therefore been recognised by the assembly software, with 19,344 contigs flagged as
268 isoformal variants of other contigs in our assembly. This is not unusual, and is in fact less than
269 that recognised in some other *de novo* assemblies (for instance, [64], where every gene
270 possessed approximately 2 "isoform" variants on average). Our samples may therefore possess
271 little in the way of genetic variability at most loci. Our reference assembly is well-assembled,
272 with a high N50 (1,113 bp) sufficient to span the full length of many protein coding genes.
273 12,997 contigs were longer than this N50 value, and 14,633 longer than 1kb in length. Given
274 that the expected complement of genes in most metazoans is around 20,000 in quantity, a
275 significant number of these are likely to be represented in these well-assembled contigs.

276 To test the completeness of our transcriptomic dataset we used the BUSCO approach[55],
277 which revealed our dataset to be remarkably complete. Of 978 BUSCO orthogroups plausibly
278 expected in any metazoan species, 916 complete BUSCOs were found (654 single copy, 262
279 duplicated, possibly reflecting allelic or isoform differences). 20 were present only as
280 fragmentary sequence, while 42 were missing. Of the 303 eukaryotic BUSCO sequences, 298
281 (98.4%) were present, 213 as single copy, 85 with duplicates and 2 as fragmentary sequence.
282 Only 3 BUSCO groups were missing. By way of comparison, the published draft of the
283 *Amphimedon queenslandica* set is missing 1.6% of the eukaryote set (5 genes), and 4.9% (49)
284 of the metazoan complement[36], and our transcriptome compares favourably with that resource.

285

286 *Annotation and Content*

287 To annotate our data, we used automated methods, including the Blast2GOPro and
288 KEGG platforms. Of 90,188 total contigs, 20,607 were given an initial annotation based on
289 BLAST results *vs*. the Refseq database. Of these contigs, 7,303 had their "best hit" to proteins
290 in the *A. queenslandica* genome. No other species had more than 1,000 contigs with "best hits"
291 to their complements, but the cnidarian *Nematostella vectensis,* a variety of invertebrate
292 deuterostomes (*Branchiostoma floridae, Saccoglossus kowalevskii* and *Strongylocentrotus*
293 *purpuratus)* and lophotrochozoan species (*Crassostrea gigas, Aplysia californica* and *Lottia*
294 *gigantea)* were the next most commonly represented metazoan species in this group. This likely
295 represents the relatively slow rate of molecular evolution in these clades when compared to
296 model species such as *Drosophila melanogaster* and *Caenhorhabditis elegans*. The diatoms
297 *Phaeodactylum tricornutum CCAP 1055/1* and *Thalassiosira pseudonana CCMP1335* were
298 observed as "best hit" species for 798 and 563 contigs respectively, which is not surprising as
299 previous work as described abundant presence of diatoms in other Antarctic sponges [65,66].
300 Annotated diatom data was not included in further analysis. No obvious bacterial, viral, human
301 or archaeal contamination was observed, although as described earlier, 797 contigs were
302 removed from our assembly by Deconseq before any further analysis.

303 More discrete annotation was then performed using Blast2GO Pro. Of the 20,607
304 contigs preliminarily annotated with Blast, 6,267 could not be annotated further. 1,389 were able
305 to be "mapped" to GO terms, but were unable to be annotated further. 12,924 contigs received
306 GO annotations, and were thus fully annotated by this process (Supplementary File 2). 27
307 additional contigs had Interproscan results and were also placed into GO categories. While the
308 expected total gene complement of a metazoan is often up to around twice this figure, our
309 annotations are nonetheless a large proportion of the expected gene count of these sponges, as
310 not all genes in even the most well-annotated genomes can be placed in GO categories.

311 We also performed KEGG annotation on our *de novo* transcriptome using the KAAS-
312 KEGG automatic annotation server, with its default BLAST settings, to understand the
313 representation of key pathways in our transcriptomic datasets. The results of these annotations
314 are provided in full as Supplementary File 3, but in general recovery was excellent with 37,313
315 genes annotated to existing KEGG terms. While sponges do not possess the full canonical
316 complements of other metazoan phyla, as these were not present in the common ancestors of
317 sponges and the broader Metazoa, we generally recover the expected gene complements of the
318 Porifera in our reference transcriptomic assembly, when mapped to the *A, queenslandica* KEGG
319 dataset. Together with BUSCO results and the raw number of annotated genes, this gives us
320 confidence in the depth of our transcriptomic resources.
321
322 *Differential Expression Results*
323 We utilised our samples to perform a differential expression analysis, aimed at
324 discerning the specific genes up- and down- regulated by exposure to acute increased
325 temperatures. It should be noted that due to experimental necessity, these are few in replicate
326 number compared to many experiments, with n=2 at best. The sample correlation matrix and
327 heatmap of relative expression for differentially expressed genes can be seen in Fig 2. While
328 replicate samples at 3 °C correlated almost identically with one another, there was slight
329 fluctuation between our 5°C replicates, perhaps due to inter-individual differential responses to
330 these challenging conditions. The large degree to which the 3 and 5 °C treatments resemble
331 one another can be seen in the matrix in Fig. 2A, where the 4 replicates show little
332 differentiation from one another (black regions of matrix). In Fig 2B, while some discreet inter-
333 sample variation is observed, note the large downregulated quadrant of the matrix at bottom
334 right, relative to high relative expression levels in the control sample at far left. Adding additional
335 replicates would increase confidence in these results, but initial findings are already compelling.

336 In general, we found a large number of genes whose expression was perturbed by heat
337 treatment at both 3 and 5 °C. Between our control and 3 °C treatments, the transcriptional
338 landscape changed significantly (with stringent criteria for significance: cut off for FDR of
339 0.0001, a min abs(log2(a/b)) change of 2), with 1,435 contigs changing significantly in
340 expression (767 upregulated, 668 downregulated at the 3 °C treatment compared to the control
341 sample). This was mirrored in our control *vs.* 5 °C comparison, where 1,230 contigs changed
342 significantly in expression (623 upregulated, 607 downregulated significantly at 5 °C compared
343 to control sample). The complete list of differentially expressed genes and further details are
344 given in Supplementary File 4. There was a significant amount of overlap between these sets. In
345 total, 322 genes were downregulated commonly in both the 3 and 5 °C samples when
346 compared to the control. 383 genes were upregulated commonly in both the 3 and 5 °C samples
347 compared to the control. These genes may be particularly interesting for further analysis, given
348 their conserved role in thermal response, and we have provided these in Supplementary File 5.
349 Contrastingly, genes that were not shared could provide specific information about responses at
350 specific temperature points, and are also made available for further study.

351 The 20 most up and down regulated genes in our control vs 3 °C and control vs 5 °C
352 samples are of clear interest in understanding thermal stress response, and can be seen in
353 Table 3. These contigs include those with similarity to heat shock proteins (*heat shock 70 B2-*
354 *like*) and ribosomal proteins (*40S ribosomal S13*, *40S ribosomal S25*), along with a large
355 number of sequences with no clear homologues in the RefSeq database, and some of less clear
356 utility (e.g. the *Plant Cadmium Resistance 3-like* family of eukaryote-conserved genes, although
357 cadmium and these genes are noted to influence the HSP pathway[67]).

358 However, very few genes (5 upregulated in 5 °C sample vs 3 °C, 11 downregulated)
359 changed significantly in expression between the 3 and 5 °C treatment. We speculate that this
360 may be due to the transcriptional machinery of this sponge already operating at maximum
361 capacity to adjust to thermal stress under the 3 °C treatment, with little extra ability to cope with

362 changes brought by further increases in temperature. Despite their small number, these genes
363 are of a variety of annotations, including *allograft inflammatory factor 1*, *ubiquitin carboxyl-*
364 *terminal hydrolase isozyme L3-like* and a *FKBP6-like* sequence, and could still be helping adjust
365 to thermal stress.
366
367 *GO over-representation analysis*
368 To gain a broad understanding of the nature of changes between our samples, we
369 tested differentially expressed genes at given temperatures as our test sets, relative to our
370 reference transcriptomes, and checked for over-representation of any gene categories using
371 Fisher's Exact Test, correcting for false discovery rate and with a cutoff p value of 0.05, as
372 integrated in Blast2GO. Our findings are based on a small number of replicates, but with a high
373 stringency criteria for assigning significance. When we compared our control to our 3 and 5 °C
374 samples a variety of statistically significant differences in contig expression were observed (Fig
375 3A,B). However, there was no difference in significant GO category expression between our 3
376 and 5 °C samples, and we infer that this is due to only minor changes between 3 and 5 °C.
377 These contigs change little in expression, as they are either “on” or “off” as a result of heat
378 stress (to a degree which may or may not be significant vs the control), but cannot be
379 modulated any further under additional stress.
380 In our control vs 3 °C samples (Fig 3A), GO category representation changed
381 significantly in a number of categories. Among the GO categories downregulated at 3 °C,
382 ‘immune response’, ‘signal transduction’ ‘serine type endopeptidase inhibitor activity’ and
383 ‘pantothenate biosynthetic pathway’ were significantly more prevalent there than in our overall
384 set. These GO terms indicate that immune responses and normal bodily maintenance may be
385 perturbed by thermal stress. In contrast, GO terms associated with ribosomes and structural
386 molecules are under-represented in this set, and therefore contigs coding for these are not

387 present in the downregulated complement after heat exposure. This is similar to the results
388 observed in Guzman and Conaco (2016)[37], as discussed further below.

389 In the GO categories upregulated at 3 °C, it again seems that normal metabolism is less
390 important for response to thermal stress than cytoskeletal responses and growth factor activity.
391 Many GO terms related to metabolic activity (e.g. 'phosphate-containing compound metabolic
392 process', 'organic substance biosynthetic process') are under-represented in these genes
393 relative to our overall library, while GO terms linked to microtubules and other cytoskeletal
394 components are significantly over-represented. Taken in concert with our GO term distribution
395 findings from downregulated genes, it seems that structural componentry is significantly
396 upregulated in response to a 3 °C heat shock.

397 Many of these findings are mirrored in our control vs 5 °C samples (Fig 3B). Four GO
398 categories, 'intracellular non-membrane-bounded organelle', 'cytoplasmic part', 'pantothenate
399 biosynthetic process' and 'signal transduction', had similar changes in abundance in
400 downregulated contigs from our 3 and 5 °C treatments compared to the control, and 'growth
401 factor activity' was significantly over-represented in contigs upregulated in response to
402 increased temperature in both samples. These have been underlined in the figure. A small
403 number of additional categories were significantly changed in our 5 °C treatment but not
404 observed in our 3 °C treatment, including a number of categories related to the biosynthesis of
405 specific molecular and cellular components. For instance, the category 'cellular macromolecule
406 biosynthetic process', is less prevalent in downregulated genes, (and therefore not itself present
407 in downregulated contigs), while 'tyrosine biosynthetic process' and other categories are over-
408 represented in up-regulated genes, reflecting underlying transcriptional activity in that regard,
409 mirroring our findings in the 3 °C treatment.

410 Previous work [37] observed the upregulation of a number of protective and signalling-
411 related pathways after heat shock. We see a similar pattern in our data, but the exact categories
412 are not seen in *Isodictya* as in their warm-water *Haliclona tubifera.* Protective mechanisms in

413 common with their results include those linked to antioxidant activity and immune response
414 activation, but do not include the toll-like receptor (TLR) signaling pathway. We also observe
415 similar broad representation of cellular ion homeostasis, messenger-mediated pathways,
416 transporter activity and microtubule-based movement-linked categories, but do not see
417 specifically calcium-mediated signaling categories, unlike those seen in their data.

418 While most of the observed categories in our data are quite broad and reflect changes in
419 a number of gene families, we can observe some quite specific changes in expression. For
420 example, the 'Tumor necrosis factor receptor binding' category is over-represented in
421 downregulated contigs at 5 °C, suggesting that this gene family is not utilised as prevalently
422 after heat shock. This finding was born out by our analysis of discrete contig up- and down-
423 regulation, as discussed in detail elsewhere in this manuscript.

424

425 *Identification of Target Signalling Cascades*

426 Given the large number of un-annotatable, novel contigs in the top 20-most differentially
427 expressed complement, we also adopted a target gene approach. From the lists of differentially
428 expressed genes, we have selected genes previously identified as playing a role in thermal
429 response for further study, as well as transcription factors with potential roles as cellular
430 mediators of this process, as can be seen in Table 4. Full details of the exact expression levels
431 of all these genes can be found in Supplementary File 4.

432 Heat shock proteins (HSPs) were prevalent in these complements. We obtained 4
433 contigs encoding a full-length sequence, corresponding to four isoforms of the gene encoding
434 for HSP70 and related proteins. These four sequences were designed as A1, A2, B and HSP-er
435 (contigs: TRINITY_DN27082_c0_g1, TRINITY_DN12545_c0_g2, TRINITY_DN24395_c0_g1,
436 and TRINITY_DN20850_c0_g1_i1, respectively). The alignment of these four putative HSP70
437 sequences from *Isodictya* sp. alongside those previously described in sponges and other

438 marine invertebrates, as well as related species, can be seen in Fig 4A (Full sequences, along
439 with alignment, Supplementary File 6).

440 The full-length sequence of these proteins showed the presence of three classical
441 signature motifs of the HSP70 family (IDLGTTYS; IIDLGGGTFDVSIL; IVLVGGSTRIPKI/VQK)
442 and the ATP/GTP binding site (Fig 4B). These four forms of HSP70 in *Isodictya* possess
443 relatively low sequence similarity (between 41.4% and 68.1% amino acid identity), and
444 individual isoforms are more closely related to some previously described forms, which may
445 indicate that HSP70A and B are ancestrally shared paralogues within the Porifera (Fig 4A). The
446 HSP70A1 and 2 genes are most closely related to the non-inducible or HSP70 cognate (HSC)
447 described for others invertebrates and vertebrates (Fig 4, Supplementary File 7). Their C-
448 terminal region contains five repeats of GGMP motif. The tetrapeptide motif GGMP repeats are
449 thought to be involved in co-chaperone-binding activities of constitutive forms[68]. HSP-A
450 sequences have a terminal motif (EEVD) indicating their cytoplasmatic localisation[69]. We do not
451 observe a clear mitochondrial-located HSP70 homologue, but HSP-er shows clear homology to
452 known endoplasmic reticulum isoforms, and clear sequence similarity to those from both
453 vertebrate and invertebrate species (Fig 4B).

454 In contrast, the isoform HSP70B showed high sequence similarity to the HSP70
455 sequence of *Xestospongia testudinaria* (75.4%) and this sequence does not possess
456 tetrapeptide repeat sequences at the C-terminal (Fig 4B). HSP-B is therefore likely the
457 "inducible" HSP gene in this species. It was up-regulated during thermal stress in this
458 experiment, listed among the most strongly-changed in expression (Table 3), and thus shares
459 characteristics of inducible isoforms previously described in other marine invertebrates such as
460 oysters (see [70]).

461 In other species, the transcription of HSPs is upregulated in response to a variety of
462 stresses[71], preventing the mis-folding and aggregation of proteins, but is notably absent in some
463 species of Antarctic fish where HSPs are constitutively turned on in some cases[67]. HSPs

464 however play a clear role in temperature response in Antarctic invertebrates [17,28,29]. Given the
465 upregulation of isoform B here, it is clear that this role is conserved in sponges, and this
466 collaborates findings previously in this phylum[37,72].

467 A number of contigs with sequence similarity to the *Tumor necrosis factor* gene family
468 were observed in our dataset, in both our upregulated and downregulated complements (n.b.
469 different contigs were present in the two complements). This finding dovetails with the
470 observation of this in the GO term over-representation analysis for contigs downregulated in our
471 5 °C treatment. This cytokine family is responsible for regulating cell death through ubiquitins,
472 but also has other functions, including the regulation of cell survival, proliferation and
473 differentiation[73,74] and the downregulation of some members of this class and upregulation of
474 others likely reflects changing cellular processes within our sponge samples of interest to those
475 studying the molecular processes underlying thermal stress responses. A single gene linked to
476 this has previously been observed to be upregulated after heat shock in sponges (*TNFAIP3*[37])
477 and the additional sequences observed here confirm the likely role of these genes in linking with
478 ubiquitins and mediating cell death and protein degradation in Porifera after heat exposure.

479 The ubiquitin related genes themselves, which play essential roles in protein degradation
480 [75,76], were well recovered in our transcriptome. Fig. 5 shows our annotation of the proteolytic
481 pathway mediated by these genes, as annotated by bi-directional best blast hit. Of genes that
482 are missing, few (12) may be truly absent from our transcriptomic resource- others are also
483 absent from *A. queenslandica* and may be absent ancestrally. Three components of this
484 pathway, TRINITY_DN31115_c0_g1 (*UBE2M; UBC12; ubiquitin-conjugating enzyme E2 M)*,
485 TRINITY_DN8356_c0_g2 (*UBC1; ubiquitin-conjugating enzyme,* aka *HIP2; huntingtin*
486 *interacting protein 2)* and TRINITY_DN25219_c0_g1 (*UBE3A; E6AP; ubiquitin-protein ligase*
487 *E3A*) were significantly upregulated in our dataset in response to heat exposure. These genes,
488 representing E2 and HECT E3 type ubiquitin conjugating genes could play specific roles in the
489 degeneration of misfolded and degenerated peptides after heat exposure, correlates well with

490 previous findings in other sponge species such as *Haliclona tubifera*[37] and the specific genes
491 are worth further consideration as potential mediators of this intracellular protective response.
492 We found a number of notable transcription factor and cell signalling pathways in our up-
493 and down-regulated gene lists, including genes in the Notch, Wnt, TNF, Sox and TGF-β
494 pathways. These genes do not need to be the most up-/down-regulated genes to have a noted
495 effect on transcription, and they represent intriguing targets as possible controllers of wider
496 processes of molecular adaptation.
497 In the most similar study to this work previously performed, in the warm water sponge *H.*
498 *tubifera* from Philippines[37], multiple *G protein coupled receptors* (GPCRs) were noted as
499 differentially expressed. These genes are posited to allow these sponges to monitor and
500 respond to their environment effectively. Several families of GPCR were noted as effected by
501 heat exposure in *H. tubifera,* including *glutamate*, *rhodopsin* and *secretin GPCRs*. None of
502 these were observed here, and therefore the light and contraction responses seen in *H. tubifera*
503 on exposure to heat stress do not seem to be conserved in *Isodictya* sp. Instead, two families of
504 orphan GPCR, *157* and *161*, were noted in our sample. The roles of these specific GPCR in
505 sponges, and in general outside the Chordata, are poorly understood, but their marked up-
506 regulation here, and the absence of the plethora of differentially expressed GPCR seen in *H.*
507 *tubifera,* suggests that these explicit genes may play specific roles in sensing or recovering from
508 heat exposure in this particular species.
509 Other examples of key cell signalling molecules identified in our study include members
510 of the Sox and Wnt gene families, notably *Sox2* and *dishevelled*. These are possibly key in the
511 control of growth response to heat stress - *Sox2* in particular is known for its role in the control
512 of pluripotency in stem cell lineages[77]. Growth will both be promoted by higher temperatures,
513 but also necessary for repairing tissue damage caused by exposure to deleterious conditions.
514 However, by far the most commonly observed family of cell signalling molecules are
515 representatives of the BMP/TGF-β pathway. A variety of contigs with blast similarity to the TGF-

516 β family of signalling molecules were observed in our differentially expressed complements,
517 including a variety of contigs annotated as bone morphogenic proteins and growth differentiation
518 factors, as well as *one eyed pinhead* and the *Tolloid metalloprotease*, which act to modulate
519 TGF-β signalling. These molecules are well known as modulators of cell fates, but any role in
520 controlling response to thermal stress has not before been noted in sponges. It is possible that
521 these molecules are acting downstream of HSP70 (as noted by Lee et al.[78]) or as part of the
522 control of molecular response to physical damage caused by high temperatures [79]. However,
523 the phylogenetic distance between the organisms where this has been studied and sponges is
524 vast, and more targeted analysis is necessary to discern the true roles of these crucial
525 molecules in this Phylum.

526 We noted the presence of the Hedgehog family of genes in our dataset, with both
527 *hedgehog precursor* (TRINITY_DN46355_c0_g1_i1) and *indian hedgehog*
528 (TRINITY_DN16789_c0_g1_i1) automatically annotated – the latter will be a slight error, as
529 *indian hedgehog* is a chordate novelty generated by duplication into paralogues, and the name
530 of this gene's annotation will derive from sequence similarity. Both contigs are therefore likely to
531 represent variants of the *hedgehog* gene itself. These could perform key roles in the regulation
532 of response to thermal stress, as they are instrumental in the fine-tuning of cell differentiation
533 responses [80]. We also note the presence of members of the Notch signalling cascade in the
534 differentially expressed complements following heat stress (e.g.TRINITY_DN30424_c0_g1_i1
535 *delta and Notch-like epidermal growth factor-related receptor-like*), with Notch-related contigs
536 downregulated after exposure to both 3 and 5 °C treatments. This likely reflects changes in how
537 cell differentiation is occurring. *Hedgehog* is often noted as a promoter of stem cell proliferation,
538 and the initial stages of heat stress response may involve this, in concert with genes such as
539 *Sox2* as noted above.

540 Marine polar organisms are interesting models for studies on the noxious effects of
541 reactive oxygen species (ROS) in cold waters. In order to cope with the formation of deleterious

542 ROS, some Antarctic marine invertebrates express enzymes and low-molecular weight
543 'scavengers' [81]. The accumulation of ROS during heat stress has been shown in previous
544 studies on Antarctic marine species [82,83], and are important to these species given the higher
545 levels of dissolved oxygen seen in cooler Antarctic waters. In our study, we have obtained a
546 good sequence coverage for several enzymes implicated in antioxidant defense
547 (Supplementary File 8). Two kinds of *superoxide dismutase* (SOD), each with different isoformal
548 variants, were identified in *Isodictya* sp. which are differentiated by the type of metal associated
549 with its active site of the enzyme: two contigs for *manganese SOD*
550 (TRINITY_DN32241_c0_g1_i1 and TRINITY_DN3280_c0_g2_i1) and four contigs for a
551 *copper/zinc SOD* (TRINITY_DN22531_ c0_g1_i1; TRINITY_DN17486_c0_g1_i1;
552 TRINITY_DN_65_c0_g1_i1; TRINITY_DN35772 _c0_g1_i1). The SOD enzyme catalyze the
553 first reaction in the dismutation of superoxide anions to water and hydrogen peroxide, before the
554 catalase enzyme transforms the hydrogen peroxide into water. This enzyme is represented by
555 three contigs that share high sequence identities with marine invertebrates such as molluscs
556 (TRINITY_DN_6504; 28381; 29099). Another transcript well represented in our dataset was the
557 *glutathione S-transferase* (*GST*) which plays a key role in the detoxification of ROS and the
558 regulation of redox balance[84]. In *Isodictya* sp. seven contigs were identified with high sequence
559 similarity to *GST*s. Previous studies have highlighted the upregulation of oxidative response and
560 antioxidant genes in response to thermal challenge in sponges, as also observed in corals[85]. In
561 our data, we observe the upregulation of several contigs with strong similarity to genes with
562 antioxidant activity, *glutaredoxin* (TRINITY_DN32811_c0_g1_i1) and *thioredoxin*
563 (TRINITY_DN32811_c0_g1_i3) [37]. We also see a contig upregulated with similarity to *oxidative*
564 *stress-induced growth inhibitor 2-like* (TRINITY_DN30012_c0_g1_i1), which could play a role in
565 downstream organisation after the detection of an oxidative stress state. It seems likely that
566 these sponges are under severe oxidative stress, and the expression of these genes reflects
567 this.

568 The presence of *allograft inflammatory factor* (TRINITY DN_12683_c0_g1_i1) in our
569 datasets was noted as these have been previously studied in Antarctic species such as the sea
570 urchin *Sterechinus neumayeri*[86]. The *Isodictya* AIF-1 sequence has a high sequence similarity
571 with that known from the sponge *Suberites domuncula,* and has two conserved calcium binding
572 motifs known as EF hands (Fig 6). AIF-1 sequences from several invertebrate and vertebrate
573 species were aligned and showed relatively high levels of conservation of the protein with both
574 groups. Phylogenetic analyses generated a congruent tree positioning the AIF from sponges in
575 a cluster with corals, distinct from vertebrate sequences. In general, *allograft inflammatory factor*
576 is known for its role in recovery from injury, rather than any specific role in protecting against
577 heat shock[87]. It has been studied in sponges previously[88], where it has been shown to act to
578 activate immunocyte-like activity, as well as in protection immediately after trauma. In the
579 Japanese oyster *Crassostrea gigas* AIF-1 stimulates hemocyte immune activation by enhancing
580 phagocytosis and expression of inflammatory cytokines[89]. Its role in the thermal stress response
581 might therefore be in protection against infection, rather than directly in tissue repair, but both
582 these processes are undoubtedly necessary under prolonged periods of acute thermal stress.
583 That Antarctic species of sponge utilize many of the same pathways to respond to
584 thermal stress as warmer water species is a useful if unsurprising finding. Their efficacy at
585 moderating response to different levels of stress does however seem to be diminished, with
586 many of these genes expressed at both 3 and 5 °C, with no significant difference in their
587 expression levels between these temperatures, as noted elsewhere in this work.
588
589 *Poriferan Responses to Temperature Stress*
590 Our results, when considered alongside previous findings in sponges from other
591 latitudes, corroborate previous conclusions regarding their transcriptomic response to acute
592 thermal stress, while suggesting specifically that cold-adapted sponges may have a limited
593 range of tolerance to increased temperatures. The changes in transcription which occurred in

594 our samples after acute short-term thermal exposure confirmed that a poriferan heat-response
595 gene list can be activated by increased temperatures, and is present in this species. The
596 complements of all 4 replicates in both conditions were very similar in both overall pattern and
597 individual composition, with differentially regulated contigs often perturbed in common at both
598 temperatures. Previous work on sponges from other latitudes[37,54] showed that HSP70 and
599 ubiquitin-related genes were specifically upregulated in response to thermal stress. Our work
600 echoes this finding, observing the activation of similar genes, such as oxidation-stress related
601 genes. The nature of the thermal stress response therefore is broadly similar to that seen in
602 other sponge species. What may differ in Antarctic species of sponge is the limits of tolerance.

603 In other sponges, the degree and type of transcriptional response is temperature-
604 dependent, with specific genes activated at different temperatures[39], where the holobiont
605 changes, or [37], where the sponge itself adapts its transcription). In contrast, our findings showed
606 few significant differences in the transcriptional response of sponges at 3 and 5 °C. We also do
607 not see changes in quite the same genes, with the absence of differentially expressed GPCR
608 and toll-like receptor (TLR) signaling genes in our data a notable difference. While previous
609 research has demonstrated that some Antarctic species are not able to respond to additional
610 increases in seawater temperature, others, such as *Nacella concinna* or *Laternula elliptica*
611 showed a classic thermal stress response with over-expression of heat stress chaperones.

612 A number of differences are noted in the response of cold water sponges here, when
613 contrasted with those of warm-water species[37]. Particularly, there does not seem to be the
614 degree of incremental change that can be seen in the transcriptional profile of warm water
615 species to increasing temperatures, and an “all or nothing” response is observed instead. Since
616 our experiment exposed sponges to acute stress, our results did not test whether cold-adapted
617 sponges could, in time, adapt to sequentially higher temperatures, and this would be a useful
618 follow-up experiment to those described here. It may be possible that with a period of
619 acclimation, sequential rises in temperature can be met by successive rounds of molecular

620 adaptation. However, at present we cannot speculate as to whether that is possible in this
621 species.
622
623 **Conclusions**
624 Climate change is a pressing issue globally, and will have a range of effects on
625 organisms worldwide. While some of these effects are more obvious or better studied than
626 others, it is some of the least-studied species which will bear the brunt of these conditions, and
627 these will have a variety of knock-on effects. Here we have studied one such species, a locally
628 abundant sponge, specialised to life in Antarctic conditions. We have generated an excellent
629 reference transcriptome from a small number of replicate samples exposed to acute thermal
630 stress, and begun to identify the molecular responses that these species will use to adjust to
631 warmer ambient temperatures. Further, we have demonstrated the clear stress that even a
632 modest increase in temperature over a short time frame (in this case, 4 hours) will place on
633 such a specialist species.
634 *Isodictya* already exercises a full measure of transcriptomic response to ambient
635 temperatures of 3 °C, and further acute stress at 5 °C leaves it little further "wriggle room".
636 Whether this stress results, in the wild, in the death of these sponges, or whether, given time,
637 they will adapt to increased temperatures, is at present uncertain. However, in the interim the
638 results presented here will allow us to begin to understand the impact of increased temperatures
639 on these still under-investigated, but nonetheless vital, species.
640
641 **Data Availability**
642 All data are available either as Supplementary Files published on Figshare for direct
643 download at https://figshare.com/s/0c90ae2e0b2a0524b036 and with DOI:
644 10.6084/m9.figshare.7048727, or from the NCBI SRA with accession number PRJNA415418.
645

Animal Ethics

No animal ethics approval procedure exists for Porifera, and no permission was required to perform the experiments described in this manuscript.

Permission to Carry Out Fieldwork

The study was conducted under the permit 806/2015 granted by the Chilean Antarctic Institute (INACH).

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Competing Interests

The authors declare they have no competing interests.

Author Contributions

MGA, MO, AF and CAC performed wet lab experiments. NJK, MGA, AR and CAC performed computational analyses. NJK, MGA and CAC wrote the main manuscript text. All authors gave final approval for publication.

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Tables

Table 1: Raw Read Data, Before and After Cleaning

Metric	Control (209)	3 °C Replicate A (117)	3 °C Replicate B (123)	5 °C Replicate A (135)	5 °C Replicate B (141)
Total Read Pairs (Initial)	25,988,569	28,513,364	26,960,296	25,793,570	26,289,401
Total Read Pairs (After Cleaning)	18,784,882	20,653,864	19,553,789	18,122,106	18,763,103
Total Bases in All Reads (Initial)	5,249,690,938	5,759,699,528	5,445,979,792	5,210,301,140	5,310,459,002
Total Bases in All Reads (After Cleaning)	3,591,119,834	3,940,343,972	3,742,325,651	3,441,581,533	3,575,958,067
GC% (Initial)	45	46	45	46	45
GC% (After Cleaning)	45	46	44	46	44
Average Q (Initial)	35.75	35.75	35.8	35.55	35.75
Average Q (After Cleaning)	37.45	37.4	37.45	37.4	37.4
% less than Q20 (Initial)	4.55	4.55	4.55	5.05	4.7
% less than Q20 (After Cleaning)	0.2	0.2	0.2	0.25	0.25

897 *Table 2:* Statistics, Reference Transcriptome Assembly

898

Number of transcripts	90,188
Number of Trinity 'genes'	70,844
Total bp in assembly	59,274,448
Max contig length (bp)	19,068
Mean contig length (bp)	657.23
Median contig length (bp)	338
% GC	43.10 %
N20 Contig Length	2,921
N50 Contig Length	1,113
# contigs in N50	12,997
Number of transcripts over 1000 bp	14,633
Transcripts w/ blast hit	20,607
Transcripts w/ GO term	12,924

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903 *Table 3:* The 20 most up/downregulated contigs in each sample cross-comparison
904

Control vs 3 degrees, Downregulated	**Annotation**	**Control vs 5 degrees, downregulated**	**Annotation**	**3 vs 5 degrees, downregulated**	**Annotation**
TRINITY_DN20177_c1_g1	---NA---	TRINITY_DN24223_c2_g2	PLANT CADMIUM RESISTANCE 3-like	TRINITY_DN23764_c0_g2	polyadenylate-binding -interacting 1-like
TRINITY_DN33508_c0_g1	---NA---	TRINITY_DN20177_c1_g1	---NA---	TRINITY_DN24223_c2_g2	PLANT CADMIUM RESISTANCE 3-like
TRINITY_DN45390_c0_g1	---NA---	TRINITY_DN8570_c0_g1	hypothetical protein	TRINITY_DN12683_c0_g1	allograft inflammatory factor 1
TRINITY_DN33508_c0_g4	---NA---	TRINITY_DN32712_c4_g3	---NA---	TRINITY_DN6163_c0_g2	ubiquitin carboxyl-terminal hydrolase isozyme L3-like
TRINITY_DN35469_c0_g1	soluble calcium-activated nucleotidase 1 isoform X1	TRINITY_DN24027_c0_g1	---NA---	TRINITY_DN23190_c0_g1	---NA---
TRINITY_DN35123_c0_g3	---NA---	TRINITY_DN40223_c0_g1	---NA---	TRINITY_DN24468_c0_g2	---NA---
TRINITY_DN19331_c1_g1	hypothetical protein	TRINITY_DN8786_c0_g1	oxysterol-binding 1-like	TRINITY_DN30615_c2_g9	---NA---
TRINITY_DN24815_c0_g1	---NA---	TRINITY_DN35469_c0_g1	soluble calcium-activated nucleotidase 1 isoform X1	TRINITY_DN8356_c0_g2	ubiquitin-conjugating enzyme E2 K-like
TRINITY_DN8271_c0_g1	---NA---	TRINITY_DN45390_c0_g1	---NA---	TRINITY_DN26078_c0_g2	COMM domain-containing 8-like
TRINITY_DN31187_c0_g1	---NA---	TRINITY_DN33246_c0_g3	---NA---	TRINITY_DN58418_c0_g1	nucleoporin GLE1-like
TRINITY_DN49200_c0_g1	---NA---	TRINITY_DN33508_c0_g4	---NA---	TRINITY_DN35203_c0_g2	---NA---
TRINITY_DN33462_c0_g1	---NA---	TRINITY_DN11747_c0_g1	hypothetical protein, partial		
TRINITY_DN4804_c0_g2	hypothetical protein TRIADDRAFT_59764	TRINITY_DN24815_c0_g1	---NA---		
TRINITY_DN23863_c0_g1	---NA---	TRINITY_DN24481_c0_g1	---NA---		
TRINITY_DN33246_c0_g3	---NA---	TRINITY_DN30570_c0_g1	replicase poly		
TRINITY_DN30570_c0_g1	---NA---	TRINITY_DN35123_c0_g3	---NA---		
TRINITY_DN26078_c0_g1	COMM domain-containing 8-like	TRINITY_DN59119_c0_g1	---NA---		
TRINITY_DN12307_c0_g1	PREDICTED: uncharacterized protein	TRINITY_DN13825_c0_g1	endoplasmic reticulum-Golgi intermediate compartment 3-like		
TRINITY_DN36675_c0_g1	---NA---	TRINITY_DN26662_c0_g1	hypothetical protein crov191		
Control vs 3 degrees, Upregulated	**Annotation**	**Control vs 5 degrees, Upregulated**	**Annotation**	**3 vs 5 degrees, upregulated**	**Annotation**
TRINITY_DN24284_c0_	hypothetical protein	TRINITY_DN24284_c0_	hypothetical protein	TRINITY_DN33462_c0_	---NA---

g3	BRAFLDRAFT_114823	g3	BRAFLDRAFT_114823	g1	
TRINITY_DN44573_c0_g1	hypothetical protein	TRINITY_DN24223_c2_g1	PLANT CADMIUM RESISTANCE 3-like	TRINITY_DN53922_c0_g3	inactive peptidyl-prolyl cis-trans isomerase FKBP6-like
TRINITY_DN18944_c0_g2	40S ribosomal S13	TRINITY_DN44573_c0_g1	hypothetical protein	TRINITY_DN23764_c0_g1	polyadenylate-binding -interacting 1-like
TRINITY_DN33508_c0_g2	---NA---	TRINITY_DN22854_c0_g2	PREDICTED: tetraspanin-7-like	TRINITY_DN58083_c0_g1	---NA---
TRINITY_DN24223_c2_g1	PLANT CADMIUM RESISTANCE 3-like	TRINITY_DN8786_c0_g2	oxysterol-binding 1-like	TRINITY_DN23863_c0_g1	---NA---
TRINITY_DN34845_c4_g12	---NA---	TRINITY_DN29050_c0_g1	hypothetical protein		
TRINITY_DN34845_c4_g3	---NA---	TRINITY_DN18944_c0_g2	40S ribosomal S13		
TRINITY_DN22854_c0_g2	PREDICTED: tetraspanin-7-like	TRINITY_DN53805_c0_g1	PREDICTED: uncharacterized protein		
TRINITY_DN34845_c4_g9	---NA---	TRINITY_DN30712_c0_g1	---NA---		
TRINITY_DN22240_c0_g4	---NA---	TRINITY_DN58083_c0_g1	---NA---		
TRINITY_DN34845_c4_g10	---NA---	TRINITY_DN26231_c0_g4	dolichyl-diphosphooligosaccharide-- glycosyltransferase 48 kDa subunit-like		
TRINITY_DN44814_c0_g1	---NA---	TRINITY_DN27017_c0_g1	---NA---		
TRINITY_DN26511_c0_g1	hypothetical protein	TRINITY_DN34845_c4_g10	---NA---		
TRINITY_DN34845_c4_g4	---NA---	TRINITY_DN24395_c0_g1	heat shock 70 B2-like		
TRINITY_DN1002_c0_g1	predicted protein	TRINITY_DN34845_c4_g4	---NA---		
TRINITY_DN23682_c0_g2	---NA---	TRINITY_DN34845_c4_g12	---NA---		
TRINITY_DN26231_c0_g4	dolichyl-diphosphooligosaccharide-- glycosyltransferase 48 kDa subunit-like	TRINITY_DN34845_c4_g3	---NA---		
TRINITY_DN2258_c0_g1	40S ribosomal S25	TRINITY_DN22240_c0_g4	---NA---		
TRINITY_DN30873_c1_g2	---NA---	TRINITY_DN26511_c0_g1	---NA---		
TRINITY_DN20209_c0_g1	---NA---	TRINITY_DN34845_c4_g9	---NA---		

907 *Table 4:* Manually identified target genes
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909

Target gene approach					
Control vs 3 degrees, Downregulated	**Annotation**	**Control vs 5 degrees, downregulated**	**Annotation**	**3 vs 5 degrees, downregulated**	**Annotation**
TRINITY_DN49841_c0_g3_i1	tumor necrosis factor ligand superfamily member 15	TRINITY_DN49841_c0_g3_i1	tumor necrosis factor ligand superfamily member 15	TRINITY_DN23764_c0_g2	polyadenylate-binding -interacting 1-like
TRINITY_DN49577_c0_g1_i1	mesoderm development candidate 1-like	TRINITY_DN49577_c0_g1_i1	mesoderm development candidate 1-like	TRINITY_DN24223_c2_g2	PLANT CADMIUM RESISTANCE 3-like
TRINITY_DN34885_c0_g2_i2	neurogenic locus notch homolog 1-like	TRINITY_DN34885_c0_g2_i2	neurogenic locus notch homolog 1-like	TRINITY_DN12683_c0_g1	allograft inflammatory factor 1
TRINITY_DN28743_c0_g1_i1	E3 ubiquitin- ligase TRIM71-like	TRINITY_DN28743_c0_g1_i1	E3 ubiquitin- ligase TRIM71-like	TRINITY_DN6163_c0_g2	ubiquitin carboxyl-terminal hydrolase isozyme L3-like
TRINITY_DN28248_c0_g1_i1	ribosome-binding 1-like	TRINITY_DN33363_c1_g1_i1	G2 M phase-specific E3 ubiquitin- ligase- partial	TRINITY_DN8356_c0_g2	ubiquitin-conjugating enzyme E2 K-like
TRINITY_DN30424_c0_g1_i1	delta and Notch-like epidermal growth factor-related receptor-like	TRINITY_DN28248_c0_g1_i1	ribosome-binding 1-like	TRINITY_DN26078_c0_g2	COMM domain-containing 8-like
TRINITY_DN31499_c1_g3_i1	calmodulin isoform X1	TRINITY_DN4806_c0_g1_i1	polycomb group RING finger 1-like	TRINITY_DN58418_c0_g1	nucleoporin GLE1-like
TRINITY_DN29988_c0_g1_i1	TGF-beta receptor type-1-like	TRINITY_DN30424_c0_g1_i1	delta and Notch-like epidermal growth factor-related receptor-like		
TRINITY_DN35391_c8_g6_i2	TNF receptor-associated factor 5-like	TRINITY_DN31499_c1_g3_i1	calmodulin isoform X1		
TRINITY_DN29288_c0_g1_i1	G- coupled receptor 161-like	TRINITY_DN35391_c8_g6_i2	TNF receptor-associated factor 5-like		
TRINITY_DN54189_c0_g2_i1	probable G- coupled receptor 157	TRINITY_DN29288_c0_g1_i1	G- coupled receptor 161-like		
		TRINITY_DN54189_c0_g2_i1	probable G- coupled receptor 157		
Control vs 3 degrees, Upregulated	**Annotation**	**Control vs 5 degrees, Upregulated**	**Annotation**	**3 vs 5 degrees, upregulated**	**Annotation**
TRINITY_DN12683_c0_g1_i1	allograft inflammatory factor 1	TRINITY_DN25219_c0_g1_i1	ubiquitin- ligase E3A-like	TRINITY_DN53922_c0_g3	inactive peptidyl-prolyl cis-trans isomerase FKBP6-like
TRINITY_DN27456_c0_g1_i1	tolloid 1	TRINITY_DN40009_c0_g1_i1	transcription factor Sox-2-like	TRINITY_DN23764_c0_g1	polyadenylate-binding -interacting 1-like
TRINITY_DN25219_c0_g1_i1	ubiquitin- ligase E3A-like	TRINITY_DN24395_c0_g1_i1	heat shock 70 B2-like		
TRINITY_DN40009_c0_g1_i1	transcription factor Sox-2-like	TRINITY_DN33837_c0_g3_i1	TNF receptor-associated factor 5-like		
TRINITY_DN24395_c0_g1_i1	heat shock 70 B2-like	TRINITY_DN34897_c0_g1_i1	tolloid 2		
TRINITY_DN16789_c0_g1_i1	indian hedgehog	TRINITY_DN29751_c0_g1_i1	growth differentiation factor 7		
TRINITY_DN33837_c0_g3_i1	TNF receptor-associated factor 5-like	TRINITY_DN29790_c0_g3_i1	ubiquitin-60S ribosomal L40		
TRINITY_DN34897_c0_g1_i1	tolloid 2	TRINITY_DN35733_c0_g1_i1	probable E3 ubiquitin-ligase partial		

TRINITY_DN29790_c0_g3_i1	ubiquitin-60S ribosomal L40	TRINITY_DN16075_c0_g1_i1	bone morphogenetic 7		
TRINITY_DN28521_c0_g1_i1	bone morphogenetic 6-like	TRINITY_DN27508_c0_g1_i1	growth differentiation factor 8-like		
TRINITY_DN8356_c0_g2_i1	ubiquitin-conjugating enzyme E2 K-like	TRINITY_DN27456_c0_g1_i1	tolloid 1		
TRINITY_DN16075_c0_g1_i1	bone morphogenetic 7	TRINITY_DN34419_c1_g1_i1	pinhead precursor		
TRINITY_DN27508_c0_g1_i1	growth differentiation factor 8-like	TRINITY_DN30012_c0_g1_i1	oxidative stress-induced growth inhibitor 2-like		
TRINITY_DN31167_c0_g1_i2	segment polarity dishevelled homolog	TRINITY_DN32735_c0_g1_i2	G- coupled receptor 161-like		
TRINITY_DN46355_c0_g1_i1	hedgehog precursor				
TRINITY_DN34419_c1_g1_i1	pinhead precursor				
TRINITY_DN19433_c0_g1_i1	MULTISPECIES: cold-shock				
TRINITY_DN32811_c0_g1_i1	glutaredoxin				
TRINITY_DN32811_c0_g1_i3	thioredoxin				
TRINITY_DN33675_c0_g2_i1	alkyl hydroperoxide reductase				

Figure Legends

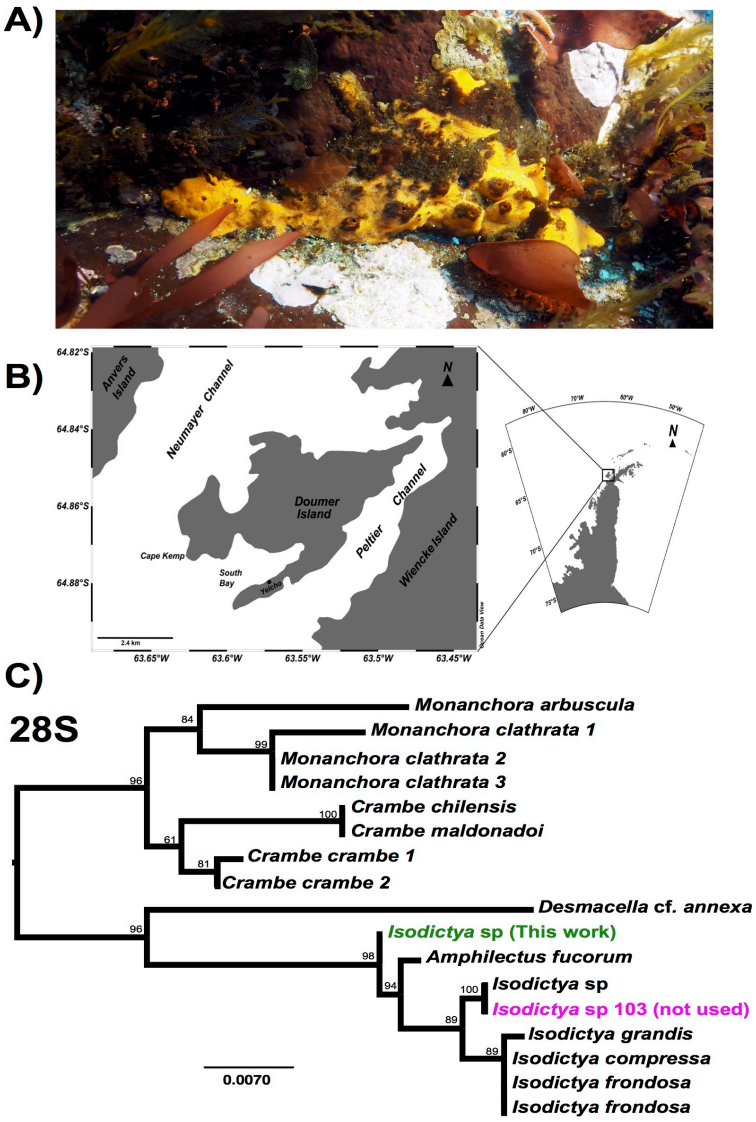


Figure 1: A) Image of *Isodictya* sp. collected at South Bay, WAP (10 m depth). B) Sample collection location, Doumer Island, WAP. C) RAxML trees of 28S rRNA under the GTR model from the *Isodictya* species mentioned in this manuscript, along with those of related species. Note the difference in placement of the samples used in the present manuscript (in green, *Isodictya* sp., this work) to that of one of the samples sequenced for this work, *Isodictya* sp. 103 (in magenta), which was excluded due to its being identity as a cryptic, but separate, species.

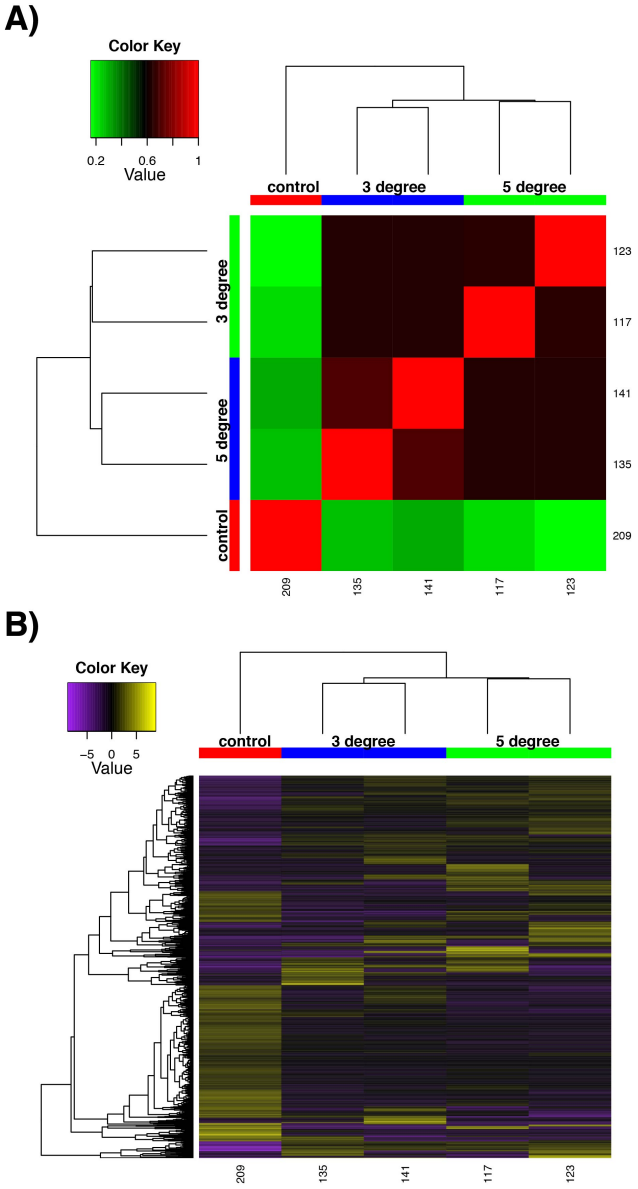
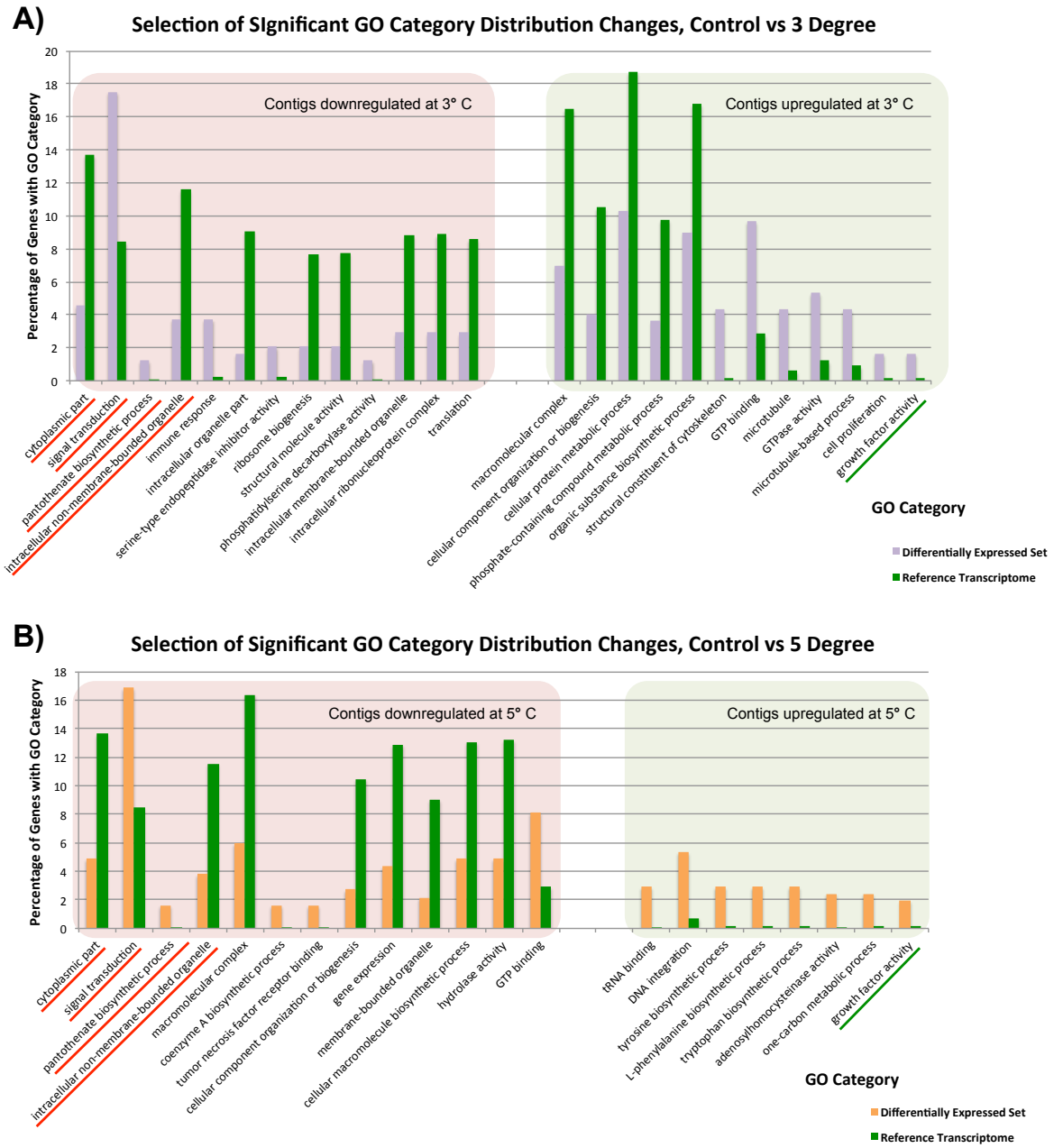


Figure 2: Differential expression analysis results performed by RSEM within the Trinity framework, with “as gene” results shown. A) is the sample correlation matrix for the five samples used in the final cross-comparison. B) shows the relative expression of each differentially expressed contig across all the samples. Note that contigs occurring uniquely in these samples are not included, as detailed in Methods. For both A and B, note the areas of the matrix shown in black hues, indicating few measurable differences between-samples.

934



935

936 **Figure 3:** Significantly differentially represented GO categories from contigs up/down regulated
937 at 3 and 5 °C compared to control sample. A) shows categories from our control vs 3 °C
938 comparison, while B) shows those from the control vs 5 °C comparison. Those categories
939 represented in both comparisons are underlined.

940

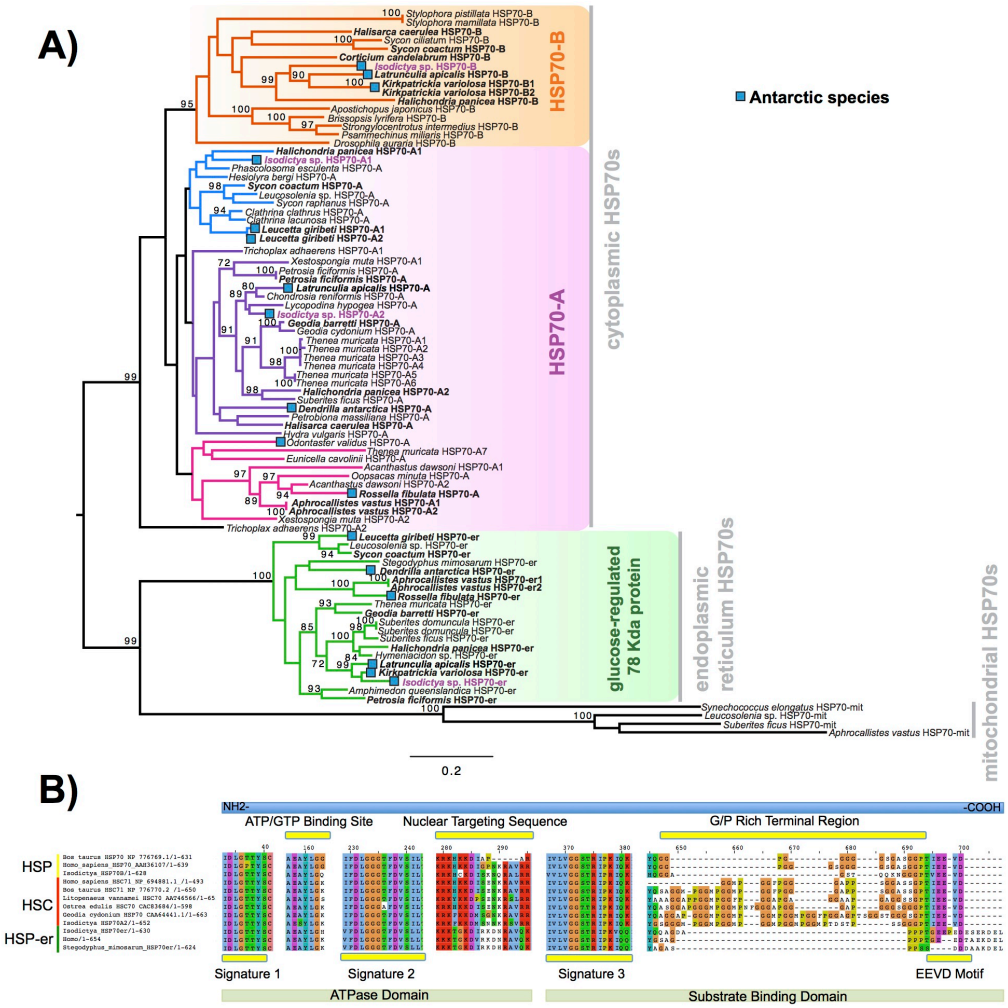


Figure 4: A) Maximum likelihood-derived phylogeny (RAxML, LG+I+G) of HSP70 sequences of known homology, together with novel *Isodictya* sequences (Names in purple). HSP70 sequences of Antarctic-resident species are bolded. Boxed as indicated on the figure are the HSP70-B, HSP70-A (HSC) and glucose-regulated 78 kDa HSP70 clades. Numbers at base of nodes indicate bootstrap support (as %ge of 1000 replicates). 5B: Multiple alignment of different HSP70 isoforms found in *Isodictya* sp. with (inducible) HSP70-B, sequence at top, HSP70-A (non-inducible heat-shock cognate) central, and below, HSP-mt sequences. These are shown alongside sequences from other species, showing the differences in domain content between isoforms. All HSPs have two main domains: the ATPase domain involved in ATP hydrolysis, and the substrate binding domain, which binds extended polypeptides, as shown at the bottom of the figure. Yellow boxes represent signature domains within HSP70 proteins. The G/P rich terminal region is prominent in HSPC (HSP-B). The EEVD sequence motif allows the cytoplasmic localisation of HSP and HSC isoforms. Accession numbers as provided in Figure, all sequences are provided, along with alignments, in Supplementary File 6.

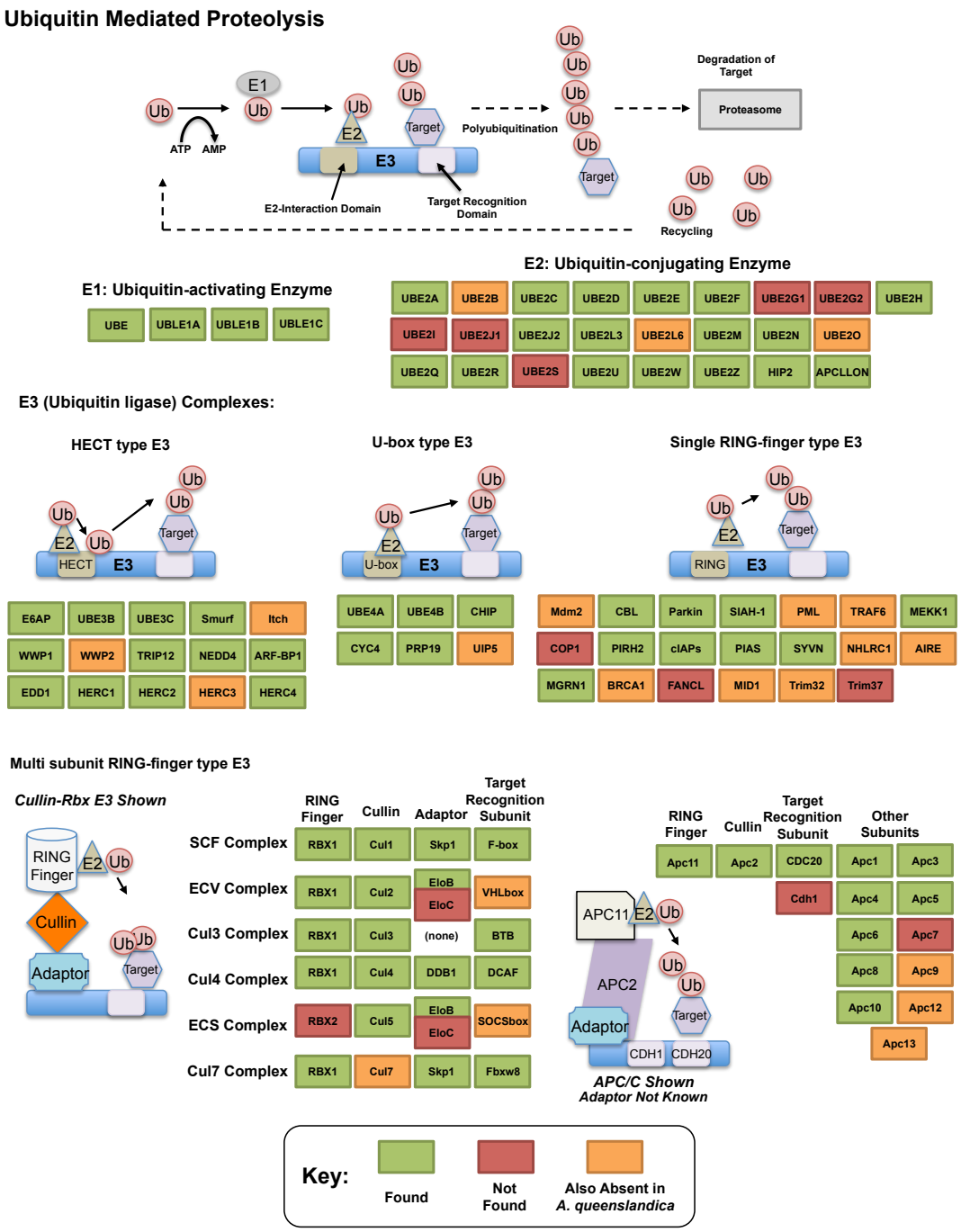
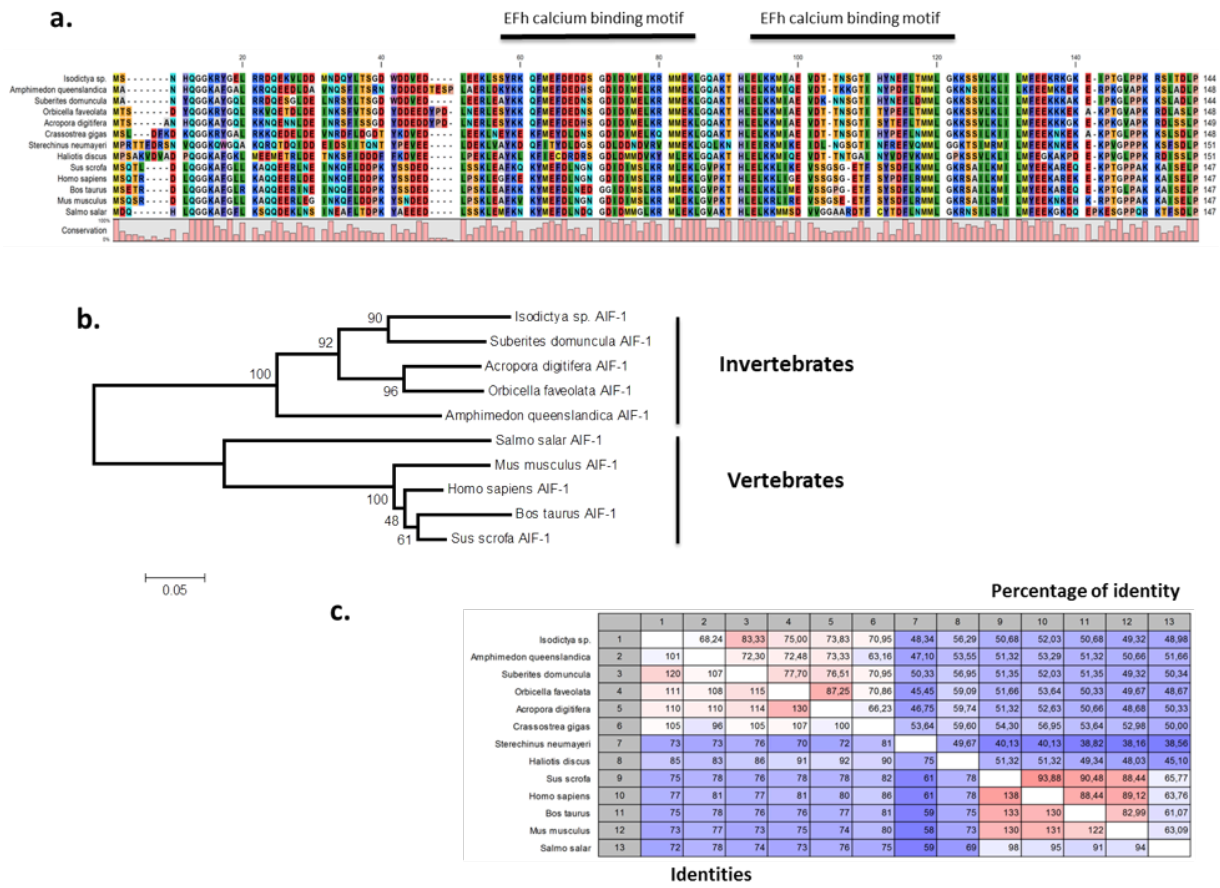


Figure 5: KEGG style map showing recovery of *Ubiquitin* mediated proteolysis genes in our resource as assessed by bi-directional best blast hit, alongside that of the sequenced genome of *Amphimedon queenslandica.* Genes noted as present in this resource indicated in green, with those absent in our transcriptome noted in orange if also missing from *A. queenslandica* (and therefore a possible poriferan absence), or red if absent from our resource only (and therefore a likely true absence).



	1	2	3	4	5	6	7	8	9	10	11	12	13
Isodictya sp. (1)		68,24	83,33	75,00	73,83	70,95	48,34	56,29	50,68	52,03	50,68	49,32	48,98
Amphimedon queenslandica (2)	101		72,30	72,48	73,33	63,16	47,10	53,55	51,32	53,29	51,32	50,66	51,66
Suberites domuncula (3)	120	107		77,70	76,51	70,95	50,33	56,95	51,35	52,03	51,35	49,32	50,34
Orbicella faveolata (4)	111	108	115		87,25	70,86	45,45	59,09	51,66	53,64	50,33	49,67	48,67
Acropora digitifera (5)	110	110	114	130		66,23	46,75	59,74	51,32	52,63	50,66	48,68	50,33
Crassostrea gigas (6)	105	96	105	107	100		53,64	59,60	54,30	56,95	53,64	52,98	50,00
Sterechinus neumayeri (7)	73	73	76	70	72	81		49,67	40,13	40,13	38,82	38,16	38,56
Haliotis discus (8)	85	83	86	91	92	90	75		51,32	51,32	49,34	48,03	45,10
Sus scrofa (9)	75	78	76	78	78	82	61	78		93,88	90,48	88,44	65,77
Homo sapiens (10)	77	81	77	81	80	86	61	78	138		88,44	89,12	63,76
Bos taurus (11)	75	78	76	76	77	81	59	75	133	130		82,99	61,07
Mus musculus (12)	73	77	73	75	74	80	58	73	130	131	122		63,09
Salmo salar (13)	72	78	74	73	76	75	59	69	98	95	91	94	

Figure 6: Alignment and phylogenetic analysis of the AIF sequences found in our transcriptome. A) The conserved two EF-hand motifs are indicated in the alignment of several AIF-1 from other invertebrate and vertebrate sequences; b) Phylogenetic tree of the AIF-1 family using the N-J method. Bootstrap values represent the frequency of appearance (expressed as a percentage) of each clade of 1000 bootstrap replicates; c) Percentage amino acid identities between AIF-1 genes from invertebrates and vertebrates. The GenBank accession numbers for the sequences are as follows: *Suberites domuncula* CAC38780; *Acropora digitifera* XP015755194; *Orbicella faveolata* XP020615825; *Amphimedon queenslandica* XP003387413; *Salmo salar* AC169994; *Mus musculus*; *Homo sapiens* P55008; *Bos taurus* NP001071547; *Sus crofa* P81076; *Crassostrea gigas* NP001292275; *Sterechinus neumayeri* ACO40483; *Haliotis discus* ABH10674.

977 **Supplementary Files:** https://figshare.com/s/0c90ae2e0b2a0524b036

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979 Supplementary File 1: Reference transcriptome assembly (.fasta, zipped)

980 Supplementary File 2: Blast2GO annotation file incorporating Annex and Interproscan results

981 (.annot, .b2g)

982 Supplementary File 3: KEGG KAAS annotation (.txt)

983 Supplementary File 4: Differential gene expression results (.txt, zip)

984 Supplementary File 5: Gene lists, overlapping in treatments (.txt)

985 Supplementary File 6: HSP70 phylogeny alignment (.phy, .fas, .nxs, zipped)

986 Supplementary File 7: Percentage identity comparison, HSP70 genes (.jpg)

987 Supplementary File 8: Antioxidant sequences

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