Warm temperatures, cool sponges: The effect of increased temperatures on the Antarctic sponge *Isodictya* sp

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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, both long-term and occasional acute temperature rises will impact species found there, and it has become crucial to understand the capacity of these species to respond to such 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 response employed by Isodictya to adjust to heat exposure.

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 in the time frame examined, 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 short-term increases in Antarctic ocean temperature on these and other species.

3   **Warm Temperatures, Cool Sponges: The Effect of Increased Temperatures on the**

4   **Antarctic Sponge *Isodictya* sp*.***

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26 **Abstract**

28 Although the cellular and molecular responses to exposure to relatively high temperatures

29 (acute thermal stress or heat shock) have been studied previously, only sparse empirical

30 evidence of how it affects cold-water species is available. As climate change becomes more

31 pronounced in areas such as the Western Antarctic Peninsula, both long-term and occasional

32 acute temperature rises will impact species found there, and it has become crucial to

33 understand the capacity of these species to respond to such thermal stress.

34 Here we use the Antarctic sponge *Isodictya* sp. to investigate how sessile organisms

35 (particularly Porifera) can adjust to acute short-term heat stress, by exposing this species to 3

36 and 5 °C for 4 hours, corresponding to predicted temperatures under high-end 2080 IPCC-

37 SRES scenarios. Assembling a *de novo* reference transcriptome (90,188 contigs, >93.7%

38 metazoan BUSCO genes) we have begun to discern the molecular response employed

39 by *Isodictya* to adjust to heat exposure.

40 Our initial analyses suggest that TGF-β, ubiquitin and hedgehog cascades are involved,

41 alongside other genes. However, the degree and type of response changed little from 3 to 5 °C

42 in the time frame examined, suggesting that even moderate rises in temperature could cause

43 stress at the limits of this organism's capacity. Given the importance of sponges to Antarctic

44 ecosystems, our findings are vital for discerning the consequences of short-term increases in

45 Antarctic ocean temperature on these and other species.

48   **Background**

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

56   The Western Antarctic Peninsula (WAP) is one of the areas of the planet which has
57   experienced some of the most significant changes in air and water temperature[9,10]. Current
58   estimates of climate change in the Antarctic suggest that the mean temperature of seawater
59   could rise by about 0.6°C (Representative Concentration Pathway 2.6) to 2.0°C (Representative
60   Concentration Pathway 8.5) (IPCC 2014)[11]. This is expected to have major implications for
61   Antarctic organisms as they are highly sensitive to environmental variation[12,13]. For this reason
62   the projected changes in water temperature constitutes a major threat to ecosystem function in
63   these waters. Such changes would modify the prevalence of key functional species, thus
64   affecting associated ecosystem processes[14]. However, we are only beginning to understand the
65   resilience of many species found in the Antarctic to rising temperature conditions [12,15,16,17], and
66   the point to which they will be affected by any changes in mean temperature, for either long or
67   short periods of time. The deleterious effects of temperature exposure in sponges from other
68   latitudes include bleaching of symbionts, tissue necrosis and death[18]. It is vital to gain this
69   information in Antarctic sponge species, so that policy decisions can be made with a full
70   understanding of the likely impacts of these changes.

71   Some sponge species have been suggested to be relatively robust to moderate changes
72   in temperature. Caribbean and Brazilian coral-reef sponges have been investigated and found
73   to survive fluctuations in ambient temperature[19,20]. A recent study, experimentally demonstrated
74   that the boreal deep-sea sponge *Geodia barretti* is able to cope with temperature rises with few
75   ill effects[21]. Other sponge species are however not so resilient [18,22,23], and both reproduction[24]
76   and filtration[25] in sponges has been shown to be affected by increases in temperature, even
77   over short periods of time. In some cases, this has led to widespread mortality [22,26].

78   We have little current understanding as to what makes some species able to cope with
79   broad temperature ranges, and it is possible that Antarctic-dwelling species may be particularly
80   vulnerable due to their specialisation for extremely cold, relatively stable temperature
81   conditions[27]. Their molecular components in particular may have altered over evolutionary time,

82   as has been observed previously in a variety of species [17], and warmer temperatures could
83   prove deleterious. The sponge *Isodictya* sp. (Fig 1A) is no exception to this. While this sponge is
84   commonly observed around the WAP and generally lives in waters with temperatures between -
85   1.8 °C [28], it is rarely found in warmer areas, with summer peaks around the WAP reaching 1.5
86   °C and 2 °C around Marguerite Bay and Palmer Archipelago respectively. Due to its ubiquity
87   and narrow normal temperature range, this sponge is an ideal model to test the consequences
88   of temperature exposure on cold-adapted species. Increasingly this sponge is exposed to
89   warmer summer temperatures across its natural range, with shallow-water temperatures
90   reaching 3 °C in some places around the WAP[29].

91   Illumina-based sequencing can reveal both subtle and broad changes in expression in
92   studies of model and non-model organisms[30]. These studies have the additional benefit of also
93   providing a suite of information on the gene sequences of the organism in question, which is
94   particularly useful for non-model species. While genomic-level datasets within the Porifera
95   remain depauperate and limit our understanding of adaptation to particular conditions, a number
96   of investigations into the transcriptomic complements of a variety of sponges have now been
97   successfully performed [31,32,33]. The transcriptional changes in response to temperature shifts
98   that occur within sponges themselves[23,34] and changes in their bacterial symbionts[18,35,36,37.38]
99   have also only recently begun to be unveiled. Some changes in gene expression revealed by
100 these studies, such as those in HSP70 expression[34,39] are easily explained with reference to
101 known metazoan responses to temperature fluctuation and heat shock. Similarly, responses
102 such as the up-regulation of genes involved in signal transduction, tissue morphogenesis, cell
103 signalling and antioxidant activity are all of clear utility to tissues as they adapt to thermal
104 stresses. What has yet to be tested thoroughly is whether these changes are mirrored in
105 extremophile species. It is possible that gene complements adapted to extremes may be less
106 able to cope with heat shock, particularly over rapid time scales.

107   Our study demonstrates that some extremophile species such as *Isodictya* sp. may be
108 able to respond to acute short term stress, but that there may be limits to the upper bounds of
109 this response. Using a small number of replicates and two "heat shock" regimes, we have been
110 able to use these findings to investigate the specific pathways used by these organisms, and
111 contrast these with previous findings in species adapted to warmer temperature regimes. The
112 sponge studied here, *Isodictya* sp. is one of the most common species in shallow-water rocky
113 reefs (<25 m depth) around Doumer Island, Palmer Archipelago, WAP (Fig 1B).

114   In this manuscript we present the first transcriptomic analysis of the effect of acute
115 warming on a cold-adapted sponge. Due to the constraints of working with polar species in

116 Antarctic conditions the numbers of replicates is low, particularly for the control specimen. This
117 limits the strength of some of the conclusions that can be drawn from this work. Nevertheless,
118 given its novelty this knowledge will be vital for understanding the impact of temperature rises
119 on Antarctic species, but also for discrete, targeted investigation of the role of specific pathways
120 in temperature adaptation in the future.

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. The study was conducted under permit 806/2015
128 granted by the Chilean Antarctic Institute (INACH). Sponges were then transferred to the
129 laboratory at Yelcho Station, where they were maintained in 140 L fiberglass tanks with
130 unfiltered, flowing seawater (approximately 0.5 °C, pumped directly from the sea floor near to
131 collection site (5 -10 m depth)) for a week to allow acclimation to laboratory conditions. Tanks
132 were covered with two layers of 1 mm shade cloth (fiberglass 50% neutral density screen) to
133 represent light levels occurring *in situ*. Relative light intensity levels and temperature in the
134 experimental tanks were measured (1 measurement every 15 minutes) during the duration of
135 experiment with a HOBO Pendant® temperature/light data logger (Onset, USA). Sponges were
136 then placed into one of three possible treatment conditions - a control tank, where samples were
137 maintained in seawater pumped directly from the sea floor at approximately 0.5 °C, or one of
138 two possible treatment temperatures, 3 and 5 °C, with water warmed in a header tank before
139 pumping into the treatment area, and subsequently maintained at a set temperature using
140 SOBO Aquarium heaters (500 watt). These temperatures were chosen in line with empirical
141 evidence of warming in the WAP[29]. Sponge samples were placed in individual treatment tanks,
142 for a total of 3 treatments and two replicates (2x control, 2x 3 °C, 2x 5 °C) although one control
143 sample was 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 cm³, 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 less than 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[40]. Low-quality regions of reads were trimmed using Trimmomatic 0.33[41] 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[42] 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[43], with two non-standard settings: a minimum contig length of 200
175   bp and *in silico* read normalisation. Clustering of isoforms was performed natively by Trinity, but
176   the assembly was not "flattened" to preserve isoformal and splice information for future work
177   DeconSeq standalone version 0.4.3[44] was used to remove contamination, with settings -i 50 -c
178   50, using pre-prepared bacterial, archean, hsref and viral databases (2,206, 155, 1 and 3,761
179   complete genomes respectively), resulting in our final reference assembly. This is available from
180   the DOI and URL below as Supplementary File 1.

181

182   *Phylogenetic Analysis*

183   28S and HSP70 sequences were extracted from our assemblies using BLASTN[45] on a

184   local server, using sequences of known orthology from Genbank as search queries. These were

185   aligned to previously published sequences downloaded from the NCBI database using MAFFT

186   [46]. HSP70 gene alignments were cured using Gblocks[47], although this was not necessary for the

187   well-conserved 28S sequence. Phylogenetic analysis was run in RAxML GUI[48] using ML +

188   Rapid Bootstrap, the GTR model (28S)/LG+I+G model (HSP70) and 1,000 bootstrap replicates.

189   Phylogenetic and molecular evolutionary analyses of AIF genes were conducted using MEGA

190   version 7.0[49]. The tree was inferred using the Neighbor-Joining method, based on the alignment

191   of the sequences using ClustalW 2.0[50] (alignment was improved using the Seaview software

192   4.6.5[51]). Resultant tree topologies were evaluated by bootstrap analyses based on 1000

193   replicates.

194

195   *Transcriptome analysis and annotation*

196   To assess the content of the transcriptome BUSCO v1.1b1[52] was run against the

197   reference transcriptome, using the eukaryotic and metazoan Basic Universal Single Copy

198   Orthologue (BUSCO) gene lists to estimate completeness. Annotation of contigs was performed

199   by translating the longest ORF for each contig using the getORF.py python script, taking the

200   longest ORF, then using standalone BLASTP[45] (cutoff *E* value 0.000001) to search against the

201   refseq protein database. This was supplemented using Blast2GO Pro[53], where full InterPro

202   scanning, mapping, annotation (including ANNEX) and enzyme code mapping was performed,

203   the complete annotations for which are available as Supplementary File 2.

204   The KAAS-KEGG automatic annotation server was used to gain an understanding of the

205   recovery of complete pathways in our transcriptome. These were generated using the online

206   tool[54] rather than as integrated into Blast2GO, due to the increased functionality of the

207   standalone server. The bi-directional best hit method was used to identify and annotate the

208   longest orfs from our contigs, with the protein sequences generated earlier used as the basis for

209   these comparisons against a range of eukaryotic species. The maps generated by this were

210   compared directly against extant ones previously generated for the sponge *Amphimedon*

211   *queenslandica,* and the complete annotations are available as Supplementary File 3.

212

213   *Differential Expression Analysis and Over-representation Analysis*

214   Comparative analyses of gene expression were performed using RSEM[55] as packaged

215   within the Trinity[43] module, using Bowtie2[56] for alignment. The three treatments (control, 3 and 5

216   °C) had their replicates (1, 2 and 2 respectively) cross-sample normalised according to Trimmed

217   Mean of M-values. The three treatments were then cross-compared using edgeR[57] within the
218   Trinity[43] perl wrapper script, according to the "classic" pairwise model with a p-value cut off for
219   FDR of 0.0001, a minimum absolute (log2(a/b)) change of 2 (i.e. 4 fold change) and a
220   dispersion setting of 0.1, deliberately over-specified to preclude false positive identification of
221   over-expression. We excluded any differentially expressed contigs where transcription was only
222   detected in a single sample of any of the five used in this analysis, prior to clustering and any
223   further analysis or display, to avoid spurious results caused by transient expression or
224   contamination of single samples. As with the dispersion estimate, the p-value cutoff and
225   minimum absolute change values are stringent, to exclude potentially artifactual changes from
226   our analysis, given the small number of replicates. The remaining differentially-expressed gene
227   lists were then targeted for further analysis. These results are provided as Supplementary File
228   4.

229   GO over-representation analysis was performed in Blast2GO Pro[53] using Fisher's Exact
230   Test, with FDR control for multiple testing, with a p-value cutoff of 0.05[58]. The test sets were the
231   annotated results for the sequences present in each of the over-represented sets derived from
232   the differential expression analysis, analysed in turn, with the reference set the full list of
233   annotated contigs, minus those in the test set.

234

235   **Results and Discussion**

236

237   *Sequencing and Read Cleaning*

238   While 5 individual samples are noted here (a single control, and two replicates for both
239   the 3 °C and 5 °C treatments) another control sample was also initially taken and sequencing
240   performed as described above. Despite being morphologically identical, our initial assays of the
241   sequences from that sample, and particularly the sequence of common molecular markers used
242   in phylogenetic comparison, including *28S rRNA* and *cytochrome c oxidase 1* (*CO1*) markers,
243   revealed that it is in fact a cryptic related species, which will be the subject of description in the
244   future (see Fig 1C). We have not included this sample in any of the analyses in this work. The
245   lack of a second control sample meant that comparisons of the treatment conditions limited
246   some conclusions, as discussed later in this manuscript.

247   Basic sequencing metrics can be seen in Table 1, alongside those after read cleaning.
248   Our initial FastQC analysis revealed the presence of low quality nucleotide sequence in the
249   second file of many pairs. Cleaning was therefore stringent, and resulted in markedly fewer, but
250   much better average quality, reads for all samples, which were then used for assembly of the

251   reference transcriptome and for differential expression analysis. GC% (which can be a crude

252   proxy for contamination or changes in expression) was even through all our samples, between

253   44 and 46%, and changed little with cleaning. A small number of over-represented sequences

254   were also initially observed in our reads, as is commonly observed in Illumina-based

255   transcriptomic analysis due to known biases in hexamer binding[59]. The difference in average

256   quality after cleaning was modest, with an average difference of around 2 in Phred score. Our

257   original reads have been uploaded to the NCBI SRA with accession number PRJNA415418.

258

259   *Assembly and Completeness*

260   Reads from all 5 samples were used to construct a reference assembly (Table 2), for

261   use in further analysis (Supplementary File 1). We checked this for potential contamination

262   using Deconseq, comparing our data to known bacterial, viral, 'archaea' (*sensu* [60], we are aware

263   of archaean paraphyly) and human genomic sequences. With very permissive settings for

264   recognition as foreign sequence (minimum thresholds for removal, 50% similarity across at least

265   50% of the contig length) 797 contigs were tagged as potential contamination. These were

266   removed from our dataset before further analysis. It should be noted, however, that particularly

267   novel bacterial sequences not yet represented in the *nr* database may still be present in our

268   data, despite the use of polyA sequencing, as these could not be represented in our removal

269   database and therefore will not have been removed. While beyond the scope of this manuscript,

270   changes in symbiont content may be vital for long-term adaptation to change.

271   Statistics related to our cleaned reference assembly can be seen in Table 2. A total of

272   90,188 contigs are present, and Trinity has automatically assigned 70,844 as independent

273   "genes". A small amount of heterozygosity or potential splice variation has therefore been

274   recognised, with 19,344 contigs flagged as isoformal variants of other contigs in our assembly.

275   This is not unusual, and is in fact less than that recognised in some other *de novo* assemblies

276   (for instance, [61], where every gene possessed approximately 2 "isoform" variants on average).

277   Our samples may therefore possess little in the way of genetic variability at most loci. Our

278   reference assembly is well-assembled, with a high N50 (1,113 bp). 12,997 contigs were longer

279   than this N50 value, and 14,633 longer than 1kb in length.

280   To test the completeness of our transcriptomic dataset we used the BUSCO approach[52],

281   which revealed our dataset to be remarkably complete. Of 978 BUSCO orthogroups plausibly

282   expected in any metazoan species, 916 complete BUSCOs were found (654 single copy, 262

283   duplicated, possibly reflecting allelic or isoform differences). 20 were present only as

284   fragmentary sequence, while 42 were missing. Of the 303 eukaryotic BUSCO sequences, 298

285   (98.4%) were present, 213 as single copy, 85 with duplicates and 2 as fragmentary sequence.

286   Only 3 BUSCO groups were missing. By way of comparison, the published draft of the

287   *Amphimedon queenslandica* set is missing 1.6% of the eukaryote set (5 genes), and 4.9% (49)

288   of the metazoan complement[33].

289

290   *Annotation and Content*

291   To annotate our data, we used automated methods, including the Blast2GOPro and

292   KEGG platforms. Of 90,188 total contigs, 20,607 were given an initial annotation based on

293   BLAST results *vs*. the Refseq database at an *E* value cutoff of 10^{-6}, chosen empirically to allow

294   maximum annotation ability without introducing artifacts. Of these contigs, 7,303 had their "best

295   hit" to proteins in the *A. queenslandica* genome. The diatoms *Phaeodactylum tricornutum CCAP*

296   *1055/1* and *Thalassiosira pseudonana CCMP1335* were observed as "best hit" species for 798

297   and 563 contigs respectively, which is not surprising as previous work as described abundant

298   presence of diatoms in other Antarctic sponges [62,63]. Annotated diatom data was not included in

299   further analysis. No obvious bacterial, viral, human or archaeal contamination was observed,

300   although as described earlier, 797 contigs were removed from our assembly by Deconseq

301   before any further analysis.

302   More discrete annotation was then performed using Blast2GO Pro. Of the 20,607

303   contigs preliminarily annotated with Blast, 6,267 could not be annotated further. 1,389 were able

304   to be "mapped" to GO terms, but were unable to be annotated further. 12,924 contigs received

305   GO annotations (Supplementary File 2). 27 additional contigs had Interproscan results and were

306   also placed into GO categories. While the expected total gene complement of a metazoan is

307   often up to around twice this figure (e.g. *Drosophila melanogaster* has around 15,500, while

308   humans contain around 20-25,000), our annotations are nonetheless a large proportion of the

309   expected gene count of these sponges. This level of annotatable data is not unusual for *de novo*

310   transcriptome assemblies, which will contain novel genes, fragments of complete genes, non

311   coding RNA and UTR sequences alongside coding sequences. It should be noted that in even

312   the most well-annotated genomes, not all genes can be placed in GO categories.

313   We also performed KEGG annotation on our *de novo* transcriptome using the KAAS-

314   KEGG automatic annotation server, with its default BLAST settings, to understand the

315   representation of key pathways in our transcriptomic datasets. The results of these annotations

316   are provided in full as Supplementary File 3, but in general recovery was excellent with 37,313

317   genes annotated to existing KEGG terms. While sponges do not possess the full canonical

318   complements of other metazoan phyla, as these were not present in the common ancestors of

319   sponges and the broader Metazoa, we generally recover the expected gene complements of the

320   Porifera in our reference transcriptomic assembly, when mapped to the *A, queenslandica* KEGG

321   dataset. Together with BUSCO results and the raw number of annotated genes, this gives us

322   confidence in the depth of our transcriptomic resources.

323

324   *Differential Expression Results*

325   We utilised our samples to perform a differential expression analysis, aimed at

326   discerning the specific genes up- and down-regulated by exposure to acute increased

327   temperatures. It should be noted that due to experimental necessity, these are few in replicate

328   number compared to many experiments, with n=2 at best. This limits the strength of conclusions

329   that can be drawn particularly between control and treatment conditions, although treatment vs

330   treatment comparisons are more robust. The sample correlation matrix and heatmap of relative

331   expression for differentially expressed genes alone can be seen in Fig 2. The large degree to

332   which the 3 and 5 °C treatments resemble one another can be seen in the matrix in Fig 2A,

333   where the 4 replicates show little differentiation from one another (black regions of matrix).

334   Within-sample variation is observed (the long branches for each terminal node on the

335   dendrogram) but little between-treatment variation is evident (see the small distances between

336   nodes leading to the individual replicates). In Fig 2B, while some discreet inter-sample variation

337   is observed, note the large downregulated quadrant of the matrix at bottom right, relative to high

338   relative expression levels in the control sample at far left. Adding additional replicates would

339   increase confidence in these results.

340   In general, we found a large number of genes whose expression was perturbed by heat

341   treatment at both 3 and 5 °C (although please note this is in comparison to a single control

342   sample). Between our control and 3 °C treatments, the transcriptional landscape changed

343   considerably. Stringent criteria were applied for significance given the single control: cut off for

344   FDR of 0.0001, a min abs(log2(a/b)) change of 2, but these results should be taken as indicative

345   rather than quantitative. 1,435 contigs were noted to change in expression (767 upregulated,

346   668 downregulated at the 3 °C treatment compared to the control sample – again note this is a

347   single control). This was mirrored in our control *vs.* 5 °C comparison, where 1,230 contigs

348   changed significantly in expression (623 upregulated, 607 downregulated significantly at 5 °C

349   compared to the single control sample). The complete list of differentially expressed genes and

350   further details are given in Supplementary File 4. There was a large amount of overlap between

351   these sets. In total, 322 genes were downregulated commonly in both the 3 and 5 °C samples

352   when compared to the control. 383 genes were upregulated commonly in both the 3 and 5 °C

353   samples compared to the control. These genes may be particularly interesting for further
354   analysis, given their conserved role in thermal response, and we have provided these in
355   Supplementary File 5. Contrastingly, genes that were not shared could provide specific
356   information about responses at specific temperature points, and are also made available for
357   further study. We re-iterate, however, that the single control sample means that these results
358   are not as well-supported as our 3 °C vs 5 °C analysis.

359   The 20 most up and down regulated genes (and thus the ones with the highest statistical
360   support, although vs a single control) in our control vs 3 °C and control vs 5 °C samples are of
361   clear interest in understanding thermal stress response, and can be seen in Table 3. These
362   contigs include those with similarity to heat shock proteins (*heat shock 70 B2-like*) and
363   ribosomal proteins (*40S ribosomal S13*, *40S ribosomal S25*), along with a large number of
364   sequences with no clear homologues in the RefSeq database, and some of less clear utility
365   (e.g. the *Plant Cadmium Resistance 3-like* family of eukaryote-conserved genes, although
366   cadmium and these genes are noted to influence the HSP pathway[64]).

367   However, very few genes (5 upregulated in 5 °C sample vs 3 °C, 11 downregulated)
368   changed significantly in expression between the 3 and 5 °C treatment. This is a more reliable
369   measurement than our control vs treatment comparisons, given the replicates for both of these
370   samples. We speculate that this may be due to the transcriptional machinery of this sponge
371   already operating at maximum capacity to adjust to thermal stress under the 3 °C treatment,
372   with little extra ability to cope with changes brought by further increases in temperature,
373   although longer exposure might also be needed to bring about more discrete transcriptional
374   changes. Despite their small number, these genes are of a variety of annotations, including
375   *allograft inflammatory factor 1*, *ubiquitin carboxyl-terminal hydrolase isozyme L3-like* and a
376   *FKBP6-like* sequence, and could still be helping adjust to thermal stress at specific temperature
377   ranges.

378

379   *GO over-representation analysis*

380   To gain a broad understanding of the nature of changes between our samples, we
381   tested differentially expressed genes at given temperatures as our test sets, relative to our
382   reference transcriptomes, and checked for over-representation of any gene categories using
383   Fisher's Exact Test, correcting for false discovery rate and with a cutoff p value of 0.05, as
384   integrated in Blast2GO. Our findings are based on the small number of replicates mentioned
385   previously, but with a high stringency criteria for assigning significance. When we compared our
386   control to our 3 and 5 °C samples a variety of statistically significant differences in contig

387   expression were observed (Fig 3A,B). Please note that as our control sample had only a single
388   replicate, these findings are not as well supported as our 3 °C vs 5 °C comparison. In the
389   comparison of the 3 °C vs 5 °C treatments, which do have replicates, there was no difference in
390   significant GO category expression between our 3 and 5 °C samples, and we infer that this is
391   due to only minor changes between 3 and 5 °C. These contigs change little in expression, and
392   from their values at 3 °C do not (or can not) alter their levels any further to adjust to the
393   additional stress at 5 °C.

394   In our single control vs duplicate 3 °C samples (Fig 3A), GO category representation
395   changed significantly in a number of categories. Among the GO categories downregulated at 3
396   °C, 'immune response', 'signal transduction' 'serine type endopeptidase inhibitor activity' and
397   'pantothenate biosynthetic pathway' were significantly more prevalent there than in our overall
398   set. These GO terms indicate that immune responses and normal bodily maintenance may be
399   perturbed by thermal stress. In contrast, GO terms associated with ribosomes and structural
400   molecules are under-represented in this set, and therefore contigs coding for these are not
401   present in the downregulated complement after heat exposure. This is similar to the results
402   observed in Guzman and Conaco (2016)[34], as discussed further below.

403   In the GO categories upregulated at 3 °C, it again seems that normal metabolism is less
404   important for response to thermal stress than cytoskeletal responses and growth factor activity.
405   Many GO terms related to metabolic activity (e.g. 'phosphate-containing compound metabolic
406   process', 'organic substance biosynthetic process') are under-represented in these genes
407   relative to our overall library, while GO terms linked to microtubules and other cytoskeletal
408   components are significantly over-represented. Taken in concert with our GO term distribution
409   findings from downregulated genes, it seems that structural componentry is significantly
410   upregulated in response to a 3 °C heat shock, and cytoskeletal proteins can be involved in
411   stress response[65].

412   Many of these findings are mirrored in our single control vs duplicate 5 °C samples (Fig
413   3B). Four GO categories, 'intracellular non-membrane-bounded organelle', 'cytoplasmic part',
414   'pantothenate biosynthetic process' and 'signal transduction', had similar changes in abundance
415   in downregulated contigs from our 3 and 5 °C treatments compared to the control, and 'growth
416   factor activity' was significantly over-represented in contigs upregulated in response to
417   increased temperature in both samples. These have been underlined in the figure. A small
418   number of additional categories were significantly changed in our 5 °C treatment but not
419   observed in our 3 °C treatment, including a number of categories related to the biosynthesis of
420   specific molecular and cellular components. For instance, the category 'cellular macromolecule

421 biosynthetic process', is less prevalent in downregulated genes, (and therefore not itself present
422 in downregulated contigs), while 'tyrosine biosynthetic process' and other categories are over-
423 represented in up-regulated genes, reflecting underlying transcriptional activity in that regard,
424 mirroring our findings in the 3 °C treatment.

425   Previous work [34] observed the upregulation of a number of protective and signalling-
426 related pathways after heat shock. We see a similar pattern in our data, but the exact categories
427 are not seen in *Isodictya* as in their warm-water *Haliclona tubifera.* Protective mechanisms in
428 common with the *Haliclona* results include those linked to antioxidant activity and immune
429 response activation, but do not include the toll-like receptor (TLR) signaling pathway. We also
430 observe similar broad representation of cellular ion homeostasis, messenger-mediated
431 pathways, transporter activity and microtubule-based movement-linked categories, but do not
432 see specifically calcium-mediated signaling categories, unlike those seen in their data.

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

440

441 *Identification of Target Genes*

442   Given the large number of genes with no gene ontology assignment in the top 20-most
443 differentially expressed complement, we also adopted a target gene approach to identify the
444 impact of heat exposure on many well-known gene families known from previous work. From
445 the lists of differentially expressed genes, we have selected genes previously identified as
446 playing a role in thermal response for further study, as well as transcription factors with potential
447 roles as cellular mediators of this process, as can be seen in Table 4. Full details of the exact
448 expression levels of all these genes can be found in Supplementary File 4.

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

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

457   The full-length sequence of these proteins showed the presence of three classical
458   signature motifs of the HSP70 family (IDLGTTYS; IIDLGGGTFDVSIL; IVLVGGSTRIPKI/VQK)
459   and the ATP/GTP binding site (Fig 4B). These four forms of HSP70 in *Isodictya* possess
460   relatively low sequence similarity (between 41.4% and 68.1% amino acid identity), and
461   individual isoforms are more closely related to some previously described forms, which may
462   indicate that HSP70A and B are ancestrally shared paralogues within the Porifera (Fig 4A). The
463   HSP70A1 and 2 genes are most closely related to the non-inducible or HSP70 cognate (HSC)
464   described for others invertebrates and vertebrates (Fig 4, Supplementary File 7). Their C-
465   terminal region contains five repeats of GGMP motif. The tetrapeptide motif GGMP repeats are
466   thought to be involved in co-chaperone-binding activities of constitutive forms[66]. HSP-A
467   sequences have a terminal motif (EEVD) indicating their cytoplasmatic localisation[67]. We do not
468   observe a clear mitochondrial-located HSP70 homologue, but HSP-er shows clear homology to
469   known endoplasmic reticulum isoforms, and clear sequence similarity to those from both
470   vertebrate and invertebrate species (Fig 4B).

471   In contrast, the isoform HSP70B showed high sequence similarity to the HSP70
472   sequence of *Xestospongia testudinaria* (75.4%) and this sequence does not possess
473   tetrapeptide repeat sequences at the C-terminal (Fig 4B). HSP-B is therefore likely the
474   "inducible" HSP gene in this species. It was up-regulated during thermal stress in this
475   experiment, listed among the most strongly-changed in expression (Table 3), and thus shares
476   characteristics of inducible isoforms previously described in other marine invertebrates such as
477   oysters and krill (see [66,68] for more details).

478   In other species, the transcription of HSPs is upregulated in response to a variety of
479   stresses[69], preventing the mis-folding and aggregation of proteins, but is notably absent in some
480   species of Antarctic fish where HSPs are constitutively turned on in some cases[67]. HSPs
481   however play a clear role in temperature response in Antarctic invertebrates[17]. Given the
482   upregulation of isoform B here, it is clear that this role is conserved in sponges, and this
483   collaborates findings previously in this phylum[37,70].

484   A number of contigs with sequence similarity to the *Tumor necrosis factor* gene family
485   were observed in our dataset, in both our upregulated and downregulated complements (n.b.
486   different contigs were present in the two complements). This finding dovetails with the
487   observation of this in the GO term over-representation analysis for contigs downregulated in our
488   5 °C treatment. This cytokine family is responsible for regulating cell death through ubiquitins,

489   but also has other functions, including the regulation of cell survival, proliferation and
490   differentiation[71,72] and the downregulation of some members of this class and upregulation of
491   others possibly reflects changing cellular processes within our sponge samples of interest to
492   those studying the molecular processes underlying thermal stress responses. A single gene
493   linked to this has previously been observed to be upregulated after heat shock in sponges
494   (*TNFAIP3*[34]) and the additional sequences observed here suggests the possible role of these
495   genes in linking with ubiquitins and mediating cell death and protein degradation in Porifera after
496   heat exposure.

497   The ubiquitin related genes themselves, which play essential roles in protein degradation
498   [73,74], were well recovered in our transcriptome. Fig 5 shows our annotation of the proteolytic
499   pathway mediated by these genes, as annotated by bi-directional best blast hit. Of genes that
500   are missing, few (12) may be truly absent from our transcriptomic resource- others are also
501   absent from *A. queenslandica* and may be absent ancestrally. Three components of this
502   pathway, TRINITY_DN31115_c0_g1 (*UBE2M; UBC12; ubiquitin-conjugating enzyme E2 M*),
503   TRINITY_DN8356_c0_g2 (*UBC1; ubiquitin-conjugating enzyme,* aka *HIP2; huntingtin*
504   *interacting protein 2)* and TRINITY_DN25219_c0_g1 (*UBE3A; E6AP; ubiquitin-protein ligase*
505   *E3A*) were significantly upregulated in our dataset in response to heat exposure. These genes,
506   representing E2 and HECT E3 type ubiquitin conjugating genes could play specific roles in the
507   degeneration of misfolded and degenerated peptides after heat exposure, correlates well with
508   previous findings in other sponge species such as *Haliclona tubifera*[34] and the specific genes
509   are worth further consideration as potential mediators of this intracellular protective response.

510   We found a number of notable transcription factor and cell signalling pathways in our up-
511   and down-regulated gene lists, including genes in the Notch, Wnt, TNF, Sox and TGF-β
512   pathways. These are sometimes recovered in heat stress studies, with the exact families used
513   varying according to study species as detailed below. These genes do not need to be the most
514   up-/down-regulated genes to have a noted effect on transcription, and they represent intriguing
515   targets as possible controllers of wider processes of molecular adaptation.

516   Other examples of key cell signalling molecules identified in our study include members
517   of the Sox and Wnt gene families, notably *Sox2* and *dishevelled*. These are possibly key in the
518   control of growth response to heat stress - *Sox2* in particular is known for its role in the control
519   of pluripotency in stem cell lineages[75]. Growth will both be promoted by higher temperatures,
520   but also necessary for repairing tissue damage caused by exposure to deleterious conditions.
521   However, by far the most commonly observed family of cell signalling molecules are
522   representatives of the BMP/TGF-β pathway. A variety of contigs with blast similarity to the TGF-

523   β family of signalling molecules were observed in our differentially expressed complements,

524   including a variety of contigs annotated as bone morphogenic proteins and growth differentiation

525   factors, as well as *one eyed pinhead* and the *Tolloid metalloprotease*, which act to modulate

526   TGF-β signalling. These molecules are well known as modulators of cell fates, but any role in

527   controlling response to thermal stress has not before been noted in sponges. It is possible that

528   these molecules are acting downstream of HSP70 (as noted by [76,77]) or as part of the control of

529   molecular response to physical damage caused by high temperatures [79]. However, the

530   phylogenetic distance between the organisms where this has been studied and sponges is vast,

531   and more targeted analysis is necessary to discern the true roles of these crucial molecules in

532   this Phylum.

533   We noted the presence of the Hedgehog family of genes in our dataset, with both

534   *hedgehog   precursor*   (TRINITY_DN46355_c0_g1_i1)   and   *indian   hedgehog*

535   (TRINITY_DN16789_c0_g1_i1) automatically annotated – the latter will be a naming artifact

536   from annotation, as *indian hedgehog* is a chordate novelty generated by duplication into

537   paralogues. Both contigs therefore represent variants of the *hedgehog* gene itself. These could

538   perform key roles in the regulation of response to thermal stress, as they are instrumental in the

539   fine-tuning of cell differentiation responses [78]. We also note the presence of members of the

540   Notch signalling cascade in the differentially expressed complements following heat stress

541   (e.g.TRINITY_DN30424_c0_g1_i1 *delta and Notch-like epidermal growth factor-related*

542   *receptor-like*), with Notch-related contigs downregulated after exposure to both 3 and 5 °C

543   treatments. This possibly reflects changes in how cell differentiation is occurring. *Hedgehog* is

544   often noted as a promoter of stem cell proliferation, and the initial stages of heat stress

545   response may involve this, in concert with genes such as *Sox2* as noted above.

546   In the most similar study to this work previously performed, in the warm water sponge *H.*

547   *tubifera* from Philippines[34], multiple *G protein coupled receptors* (GPCRs) were noted as

548   differentially expressed. In that experiment, sponges with a normal growth temperature of 29 °C

549   were exposed to 32°C and 34°C for between 4 and 12 hours. GPCR genes are posited to allow

550   these sponges to monitor and respond to their environment effectively. Several families of

551   GPCR were noted as effected by heat exposure in *H. tubifera,* including *glutamate*, *rhodopsin*

552   and *secretin GPCRs*. None of these were observed here, and therefore the light and contraction

553   responses seen in *H. tubifera* on exposure to heat stress do not seem to be conserved in

554   *Isodictya* sp. Instead, two families of orphan GPCR, *157* and *161*, were noted in our sample.

555   The roles of these specific GPCR in sponges, and in general outside the Chordata, are poorly

556   understood, but their marked up-regulation here, and the absence of the plethora of differentially

557   expressed GPCR seen in *H. tubifera,* suggests that these explicit genes may play specific roles
558   in sensing or recovering from heat exposure in this particular species.

559   Marine polar organisms are interesting models for studies on the noxious effects of
560   reactive oxygen species (ROS) in cold waters. In order to cope with the formation of deleterious
561   ROS, some Antarctic marine invertebrates express enzymes and low-molecular weight
562   'scavengers' [79]. The accumulation of ROS during heat stress has been shown in previous
563   studies on Antarctic marine species [80,81], and are important to these species given the higher
564   levels of dissolved oxygen seen in cooler Antarctic waters. In our study, we have obtained a
565   good sequence coverage for several enzymes implicated in antioxidant defense
566   (Supplementary File 8). Two kinds of *superoxide dismutase* (SOD), each with different isoformal
567   variants, were identified in *Isodictya* sp. which are differentiated by the type of metal associated
568   with its active site of the enzyme: two contigs for *manganese SOD*
569   (TRINITY_DN32241_c0_g1_i1 and TRINITY_DN3280_c0_g2_i1) and four contigs for a
570   *copper/zinc SOD* (TRINITY_DN22531_ c0_g1_i1; TRINITY_DN17486_c0_g1_i1;
571   TRINITY_DN_65_c0_g1_i1; TRINITY_DN35772 _c0_g1_i1). The SOD enzyme catalyze the
572   first reaction in the dismutation of superoxide anions to water and hydrogen peroxide, before the
573   catalase enzyme transforms the hydrogen peroxide into water. This enzyme is represented by
574   three contigs that share high sequence identities with marine invertebrates such as molluscs
575   (TRINITY_DN_6504; 28381; 29099). Another transcript well represented in our dataset was a
576   *glutathione S-transferase* (*GST*) which plays a key role in the detoxification of ROS and the
577   regulation of redox balance[82]. In *Isodictya* sp. seven contigs were identified with high sequence
578   similarity to *GST*s, a complex family of genes with multiple intracellular functions. Previous
579   studies have highlighted the upregulation of oxidative response and antioxidant genes in
580   response to thermal challenge in sponges, as also observed in corals[83]. In our data, we observe
581   the upregulation of several contigs with strong similarity to genes with antioxidant activity,
582   *glutaredoxin* (TRINITY_DN32811_c0_g1_i1) and *thioredoxin* (TRINITY_DN32811_c0_g1_i3) [34].
583   We also see a contig upregulated with similarity to *oxidative stress-induced growth inhibitor 2-*
584   *like* (TRINITY_DN30012_c0_g1_i1), which could play a role in downstream organisation after
585   the detection of an oxidative stress state. It seems possible that these sponges are under
586   severe oxidative stress, and the expression of these genes reflects this, although the
587   experiment was stopped before any outward signs of damage (e.g. necrosis) were observed.

588   The presence of *allograft inflammatory factor* (TRINITY DN_12683_c0_g1_i1) in our
589   datasets was noted as these have been previously studied in Antarctic species such as the sea
590   urchin *Sterechinus neumayeri*[84]. The full *Isodictya* AIF-1 sequence was recovered, and has a

591 high sequence similarity with that known from the sponge *Suberites domuncula,* and has two
592 conserved calcium binding motifs known as EF hands (Fig 6). AIF-1 sequences from several
593 invertebrate and vertebrate species were aligned and showed relatively high levels of
594 conservation of the protein with both groups. Phylogenetic analyses generated a congruent tree
595 positioning the AIF from sponges in a cluster with corals, distinct from vertebrate sequences. In
596 general, *allograft inflammatory factor* is known for its role in recovery from injury, rather than any
597 specific role in protecting against heat shock[85]. It has been studied in sponges previously[86],
598 where it has been shown to act to activate immunocyte-like activity, as well as in protection
599 immediately after trauma. In the Japanese oyster *Crassostrea gigas* AIF-1 stimulates hemocyte
600 immune activation by enhancing phagocytosis and expression of inflammatory cytokines[87]. Its
601 role in the thermal stress response might therefore be in protection against infection, rather than
602 directly in tissue repair, but both these processes are undoubtedly necessary under prolonged
603 periods of acute thermal stress.

604   That Antarctic species of sponge utilize many of the same pathways to respond to
605 thermal stress as warmer water species is a useful if unsurprising finding. Their efficacy at
606 moderating response to different levels of stress does however seem to be diminished, with
607 many of these genes expressed at both 3 and 5 °C, with no significant difference in their
608 expression levels between these temperatures, as noted elsewhere in this work.

609

610 *Poriferan Responses to Temperature Stress*

611   Our results, when considered alongside previous findings in sponges from other
612 latitudes, corroborate previous conclusions regarding their transcriptomic response to acute
613 thermal stress, while suggesting specifically that cold-adapted sponges may have a limited
614 range of tolerance to increased temperatures. The changes in transcription which occurred in
615 our samples after acute short-term thermal exposure suggested that a poriferan heat-response
616 is activated by increased temperatures, and is present in this species. The complements of all 4
617 replicates in both 3 °C vs 5 °C conditions were very similar in both overall pattern and individual
618 composition, with differentially regulated contigs often perturbed in common at both
619 temperatures. Previous work on sponges from other latitudes[34,51] showed that HSP70 and
620 ubiquitin-related genes were specifically upregulated in response to thermal stress. Our work
621 echoes this finding, observing the activation of similar genes, such as oxidation-stress related
622 genes. The nature of the thermal stress response therefore is broadly similar to that seen in
623 other sponge species. What may differ in Antarctic species of sponge is the limits of tolerance.

624   In other sponges, the degree and type of transcriptional response is temperature-
625   dependent, with specific genes activated at different temperatures[36], where the holobiont
626   changes, or [34], where the sponge itself adapts its transcription). In contrast, our findings showed
627   few significant differences in the transcriptional response of sponges at 3 and 5 °C (where we
628   have replicate samples, and can thus be confident of the veracity of these results). We also do
629   not see changes in quite the same genes, with the absence of differentially expressed GPCR
630   and toll-like receptor (TLR) signaling genes in our data a notable difference. While previous
631   research has demonstrated that some Antarctic species are not able to respond to additional
632   increases in seawater temperature, others, such as *Nacella concinna* or *Laternula elliptica*
633   showed a classic thermal stress response with over-expression of heat stress chaperones.

634   A number of differences are noted in the response of cold water sponges here, when
635   contrasted with those of warm-water species[34]. Particularly, there does not seem to be the
636   degree of incremental change that can be seen in the transcriptional profile of warm water
637   species on exposure to increasing temperatures., at least in the time frame examined here.
638   Since our experiment exposed sponges to acute stress, our results did not test whether cold-
639   adapted sponges could, in time, adapt to sequentially higher temperatures, and this would be a
640   useful follow-up experiment to those described here. It may be possible that with a period of
641   acclimation, sequential rises in temperature can be met by successive rounds of molecular
642   adaptation. However, at present we cannot speculate as to whether that is possible in this
643   species.

644

645   **Conclusions**

646   Climate change is a pressing issue globally, and will have a range of effects on
647   organisms worldwide, involving both long-term and acute exposure to increased temperatures.
648   While some of these effects are more obvious or better studied than others, it is some of the
649   least-studied species which will bear the brunt of these conditions, and these will have a variety
650   of knock-on effects. Here we have studied one such species, a locally abundant sponge,
651   specialised to life in Antarctic conditions. We have generated a reference transcriptome from a
652   small number of replicate samples exposed to acute thermal stress, and identified the molecular
653   responses that these species use to adjust to these conditions. Further, we have demonstrated
654   the clear stress that even a modest increase in temperature over a short time frame (in this
655   case, 4 hours) will place on such a specialist species.

656   Using this data, we have made several comparisons between a single control sample
657   and replicate 3 °C and 5 °C heat shock samples. Comparisons between our replicate samples

658 indicate *Isodictya* already exercises a full measure of transcriptomic response to ambient
659 temperatures of 3 °C, and further stress at 5 °C leaves it little further "wriggle room", at least
660 when considering acute responses such as those tested here. Whether this stress results, in the
661 wild, in the death of these sponges, or whether, given time, they will adapt to increased
662 temperatures, is at present uncertain. However, in the interim the results presented here will
663 allow us to begin to understand the impact of increased temperatures on these still under-
664 investigated, but nonetheless vital, species.

665

666 **Animal Ethics**

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

669

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680

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Tables

918

919   *Table 1:* Raw Read Data, Before and After Cleaning

920   *Table 2:* Statistics, Reference Transcriptome Assembly

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

922   *Table 4:* Manually identified target genes, identified from BLAST based annotation

923   (BLAST2GO).

925 **Figure Legends**

926

927 **Figure 1:** A) Image of *Isodictya* sp. collected at South Bay, WAP (10 m depth). B) Sample

928 collection location, Doumer Island, WAP. C) RAxML trees of 28S rRNA under the GTR model

929 from the *Isodictya* species mentioned in this manuscript, along with those of related species.

930 Note the difference in placement of the samples used in the present manuscript (in green,

931 *Isodictya* sp., this work) to that of one of the samples sequenced for this work, *Isodictya* sp. 103

932 (in magenta), which was excluded due to its being identity as a cryptic, but separate, species.

933

934 **Figure 2:** Differential expression analysis results performed by RSEM/edgeR within the Trinity

935 framework, with "as gene" results shown. A) is the sample correlation matrix for the five samples

936 used in the final cross-comparison. B) shows the relative expression of each differentially

937 expressed contig across all the samples. Note that contigs occurring uniquely in these samples

938 are not included, as detailed in Methods. For both A and B, note the areas of the matrix shown

939 in black hues, indicating few measurable differences between-samples.

940

941 **Figure 3:** Significantly differentially represented GO categories from contigs up/down regulated

942 at 3 and 5 °C compared to control sample. A) shows categories from our control vs 3 °C

943 comparison, while B) shows those from the control vs 5 °C comparison. Those categories

944 represented in both comparisons are underlined.

945

946 **Figure 4:** A) Maximum likelihood-derived phylogeny (RAxML, LG+I+G) of HSP70 sequences of

947 known homology, together with novel *Isodictya* sequences (Names in purple). HSP70

948 sequences of Antarctic-resident species are bolded. Boxed as indicated on the figure are the

949 HSP70-B, HSP70-A (HSC) and glucose-regulated 78 kDa HSP70 clades. Numbers at base of

950 nodes indicate bootstrap support (as %ge of 1000 replicates). 5B: Multiple alignment of different

951 HSP70 isoforms found in *Isodictya* sp. with (inducible) HSP70-B, sequence at top, HSP70-A

952 (non-inducible heat-shock cognate) central, and below, HSP-mt sequences. These are shown

953 alongside sequences from other species, showing the differences in domain content between

954 isoforms. All HSPs have two main domains: the ATPase domain involved in ATP hydrolysis,

955 and the substrate binding domain, which binds extended polypeptides, as shown at the bottom

956 of the figure. Yellow boxes represent signature domains within HSP70 proteins. The G/P rich

957 terminal region is prominent in HSPC (HSP-B). The EEVD sequence motif allows the

958 cytoplasmic localisation of HSP and HSC isoforms. Accession numbers as provided in Figure,

959 all sequences are provided, along with alignments, in Supplementary File 6.

960

961 **Figure 5:** KEGG style map showing recovery of *Ubiquitin* mediated proteolysis genes in our

962 resource as assessed by bi-directional best blast hit, alongside that of the sequenced genome

963 of *Amphimedon queenslandica.* Genes noted as present in this resource indicated in green, with

964 those absent in our transcriptome noted in orange if also missing from *A. queenslandica* (and

965 therefore a possible poriferan absence), or red if absent from our resource only (and therefore a

966 likely true absence). A) Canonical Ubiquitin mediated proteolysis. B) Alternative forms of E3

967 (Ubiquitin ligase) complexes.

968

969 **Figure 6:** Alignment and phylogenetic analysis of the AIF sequences found in our

970 transcriptome. A) The conserved two EF-hand motifs are indicated in the alignment of several

971 AIF-1 from other invertebrate and vertebrate sequences; b) Phylogenetic tree of the AIF-1 family

972 using the N-J method. Bootstrap values represent the frequency of appearance (expressed as a

973 percentage) of each clade of 1000 bootstrap replicates; c) Percentage amino acid identities

974 between AIF-1 genes from invertebrates and vertebrates. The GenBank accession numbers for

975 the sequences are as follows: *Suberites domuncula* CAC38780; *Acropora digitifera*

976 XP015755194; *Orbicella faveolata* XP020615825; *Amphimedon queenslandica* XP003387413;

977 *Salmo salar* AC169994; *Mus musculus*; *Homo sapiens* P55008; *Bos taurus* NP001071547; *Sus*

978 *crofa* P81076; *Crassostrea gigas* NP001292275; *Sterechinus neumayeri* ACO40483; *Haliotis*

979 *discus* ABH10674.

980

981

Figure 1

Image of *Isodictya*, sample site and phylogeny

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.

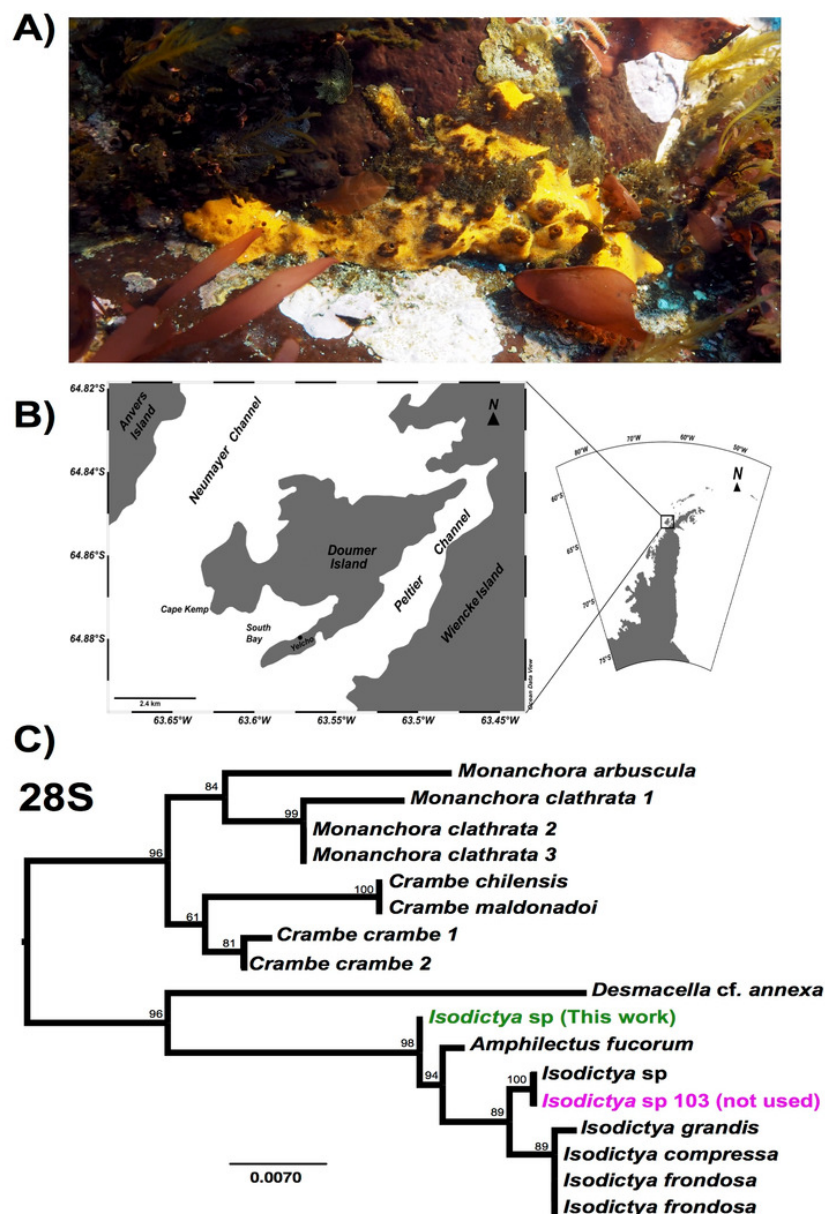
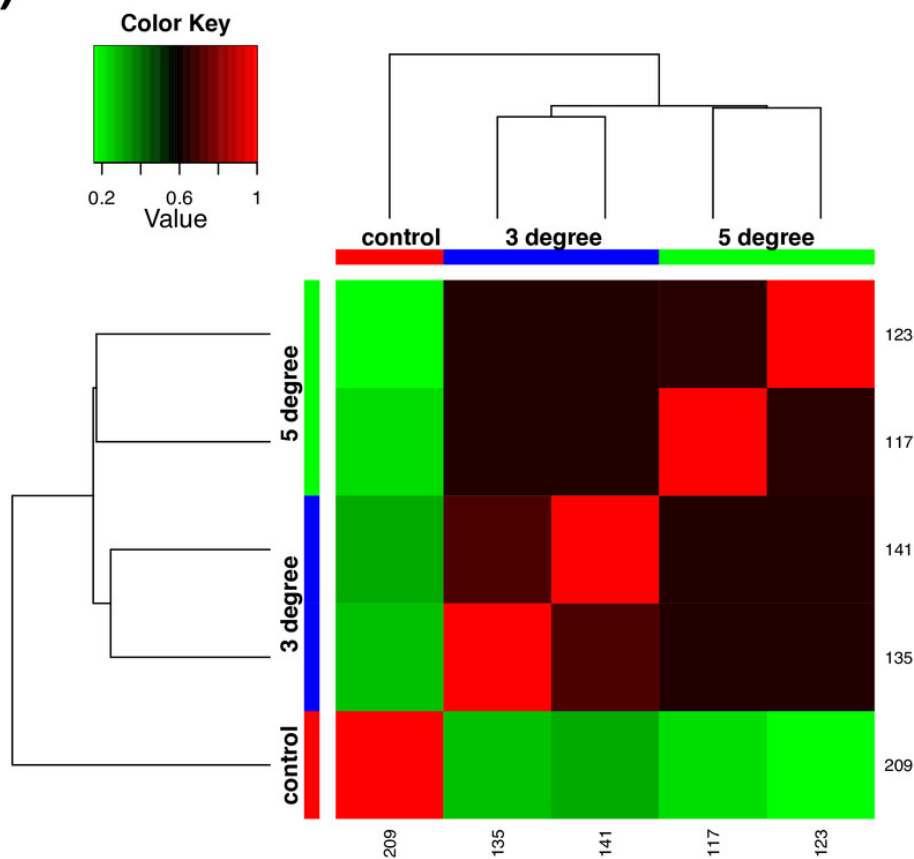



Figure 2

Differential expression results

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

A)

B)

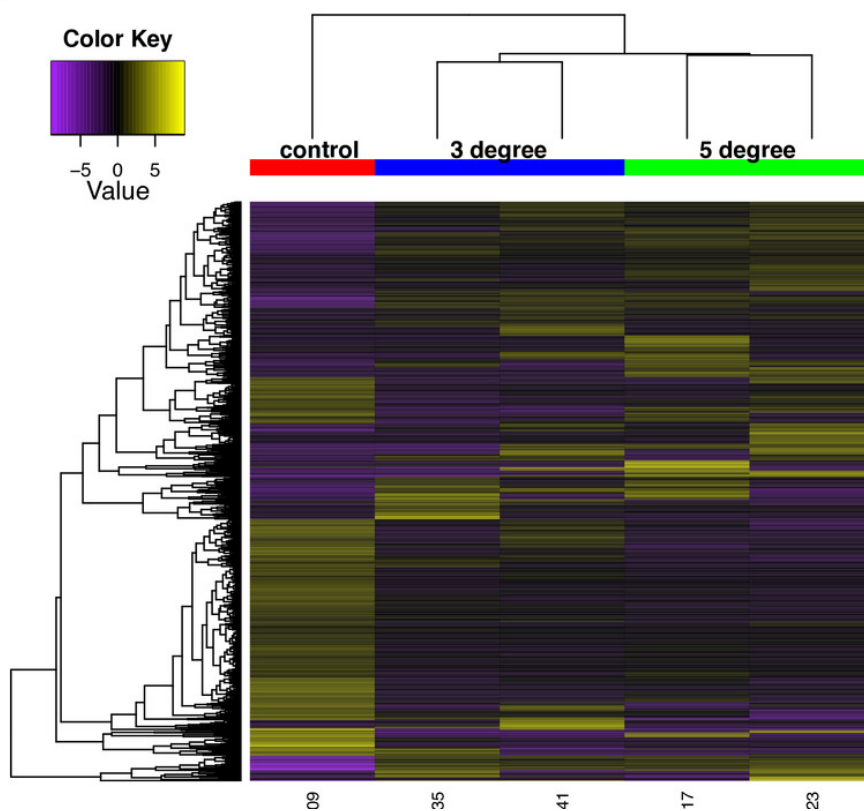



Figure 3

Significantly differentially represented GO categories

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

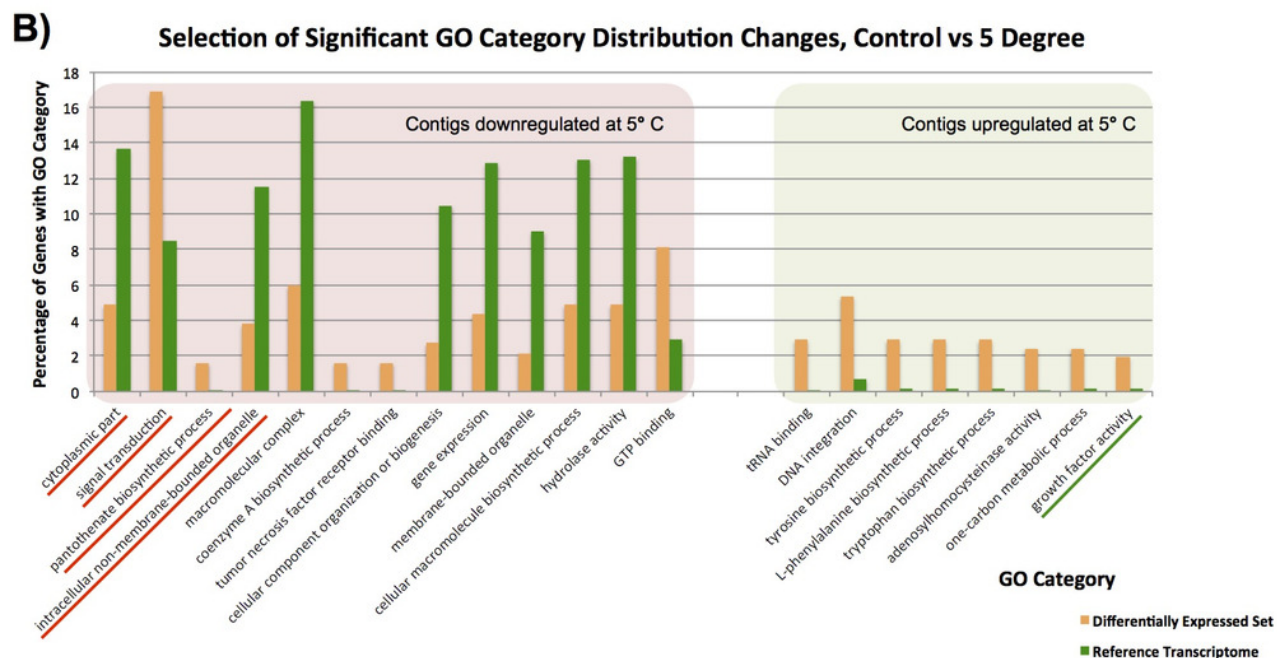
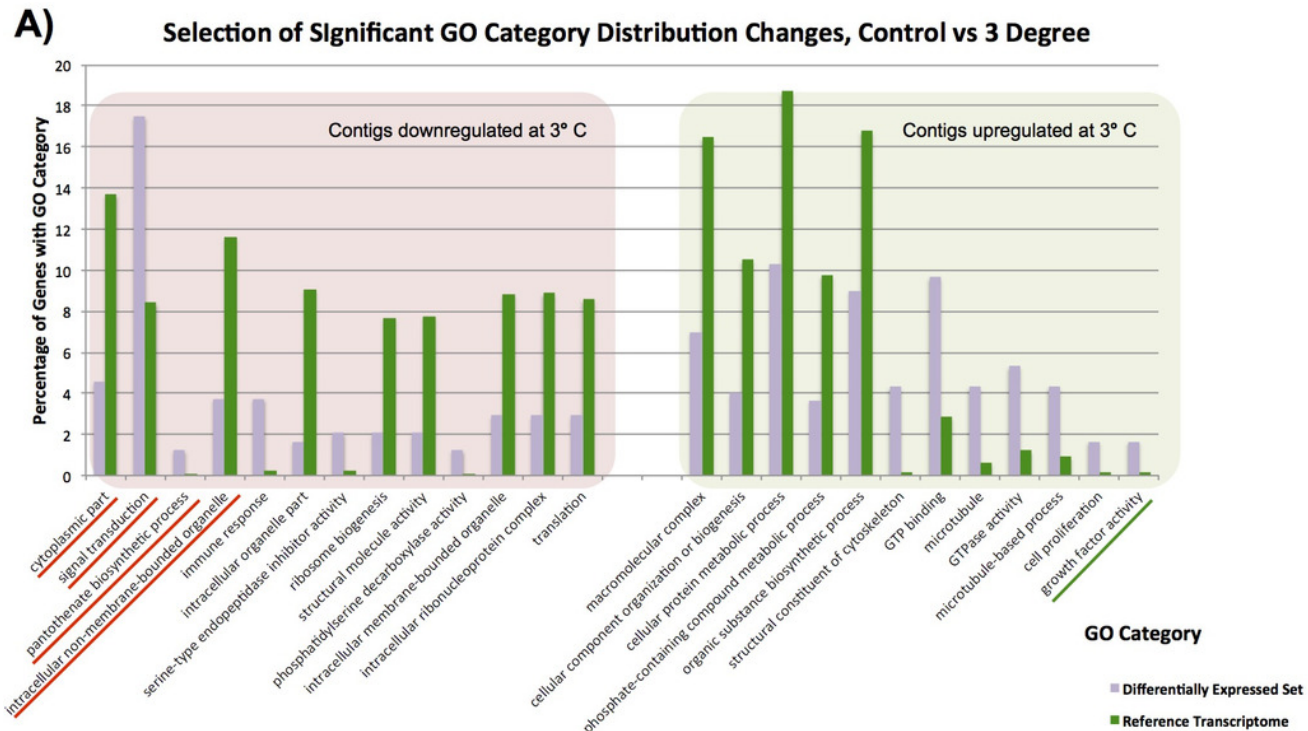



Figure 4

HSP phylogeny and alignment

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.

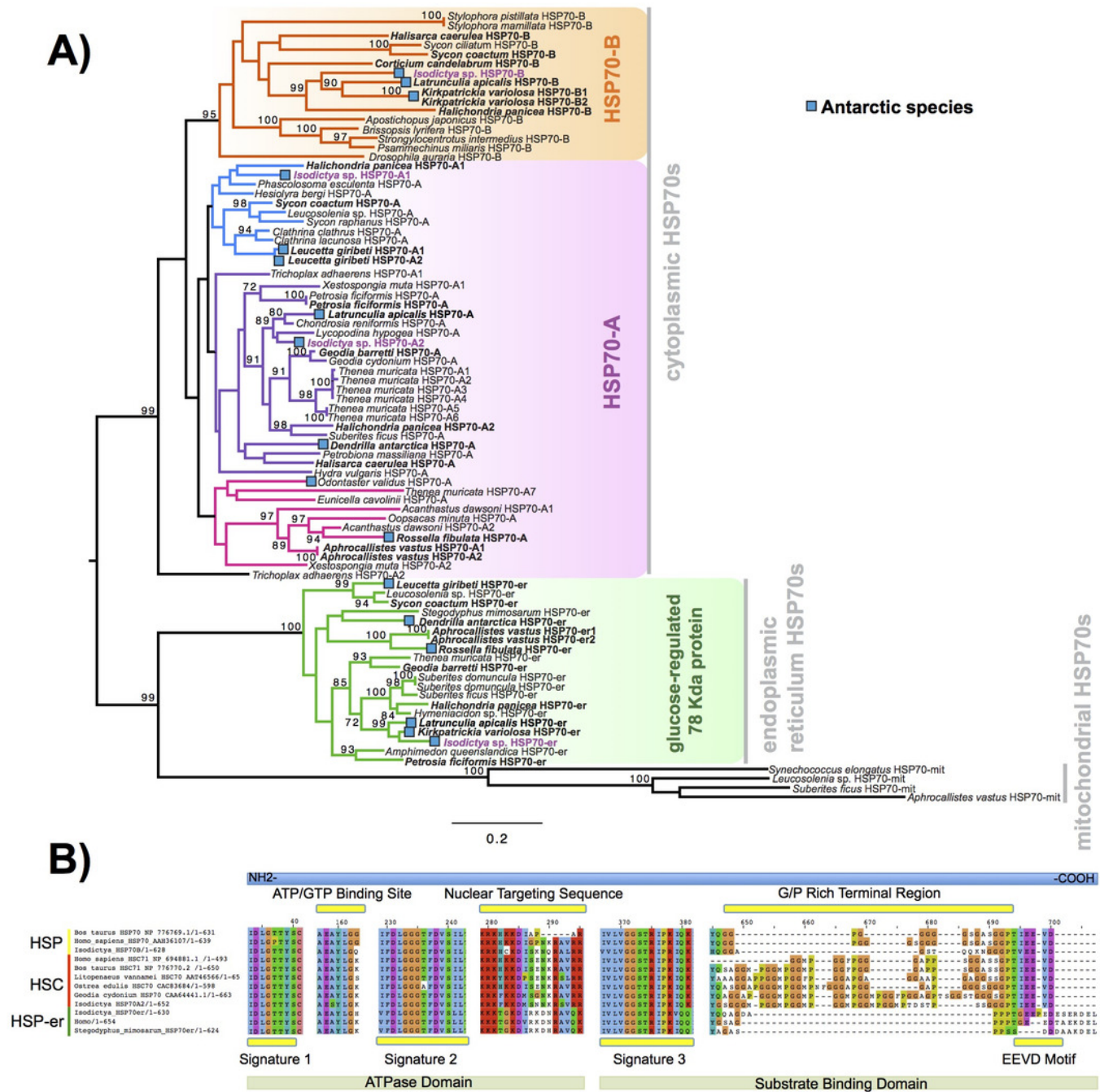



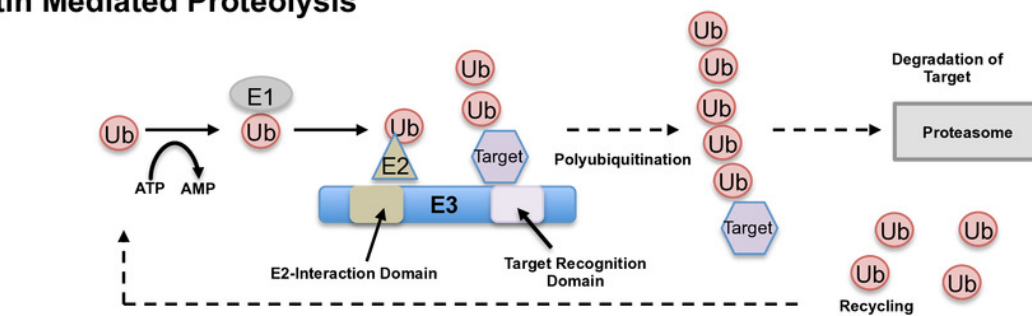
Figure 5

Ubiquitin pathway recovery

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).

Ubiquitin Mediated Proteolysis

A)

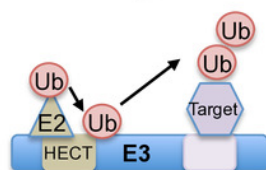
(Figure A: schematic of ubiquitin mediated proteolysis pathway showing Ub activation via ATP/AMP by E1, transfer to E2, E3 with E2-Interaction Domain and Target Recognition Domain, Polyubiquitination, Degradation of Target by Proteasome, and Recycling.)

E1: Ubiquitin-activating Enzyme

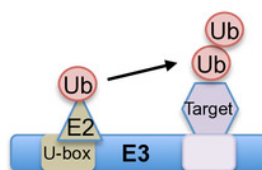
UBE	UBLE1A	UBLE1B	UBLE1C

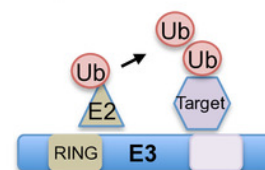
E2: Ubiquitin-conjugating Enzyme

UBE2A	UBE2B	UBE2C	UBE2D	UBE2E	UBE2F	UBE2G1	UBE2G2	UBE2H
UBE2I	UBE2J1	UBE2J2	UBE2L3	UBE2L6	UBE2M	UBE2N	UBE2O	
UBE2Q	UBE2R	UBE2S	UBE2U	UBE2W	UBE2Z	HIP2	APCLLON	

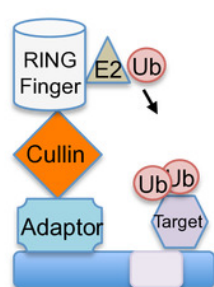
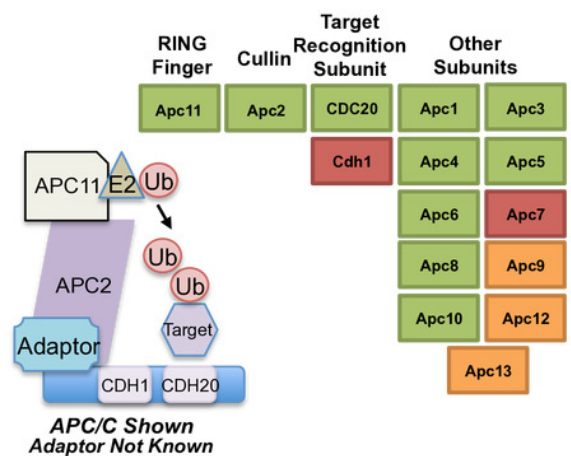
B) E3 (Ubiquitin ligase) Complexes:

HECT type E3

E6AP	UBE3B	UBE3C	Smurf	Itch
WWP1	WWP2	TRIP12	NEDD4	ARF-BP1
EDD1	HERC1	HERC2	HERC3	HERC4

U-box type E3

UBE4A	UBE4B	CHIP
CYC4	PRP19	UIP5

Single RING-finger type E3

Mdm2	CBL	Parkin	SIAH-1	PML	TRAF6	MEKK1
COP1	PIRH2	cIAPs	PIAS	SYVN	NHLRC1	AIRE
MGRN1	BRCA1	FANCL	MID1	Trim32	Trim37	

Multi subunit RING-finger type E3

Cullin-Rbx E3 Shown

	RING Finger	Cullin	Adaptor	Target Recognition Subunit
SCF Complex	RBX1	Cul1	Skp1	F-box
ECV Complex	RBX1	Cul2	EloB / EloC	VHLbox
Cul3 Complex	RBX1	Cul3	(none)	BTB
Cul4 Complex	RBX1	Cul4	DDB1	DCAF
ECS Complex	RBX2	Cul5	EloB / EloC	SOCSbox
Cul7 Complex	RBX1	Cul7	Skp1	Fbxw8

APC/C Shown
Adaptor Not Known

RING Finger	Cullin	Target Recognition Subunit	Other Subunits	
Apc11	Apc2	CDC20	Apc1	Apc3
		Cdh1	Apc4	Apc5
			Apc6	Apc7
			Apc8	Apc9
			Apc10	Apc12
			Apc13	

Key:

Found (green)	Not Found (red)	Also Absent in *A. queenslandica* (orange)

Figure 6

Figure 6: Alignment and phylogenetic analysis of the AIF sequences found in our transcriptome.

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.

a.

b.

c.

Percentage of identity

		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	

Identities

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

Statistics, Reference Transcriptome Assembly

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

The 20 most up/downregulated contigs in each sample cross-comparison

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_g3	hypothetical protein BRAFLDRAFT_114823	TRINITY_DN24284_c0_g3	hypothetical protein BRAFLDRAFT_114823	TRINITY_DN33462_c0_g1	---NA---

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---		

Manually identified target genes

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				

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