Stressed out underground?: Transcriptomics illuminates the genome-wide response to heat stress in surface and subterranean diving beetles

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Abstract

Subterranean habitats are environmentally stable with respect to temperature, humidity, and the absence of light. The transition to a subterranean lifestyle might therefore be expected to cause considerable shifts in an organism's physiology; here, we investigate how subterranean colonisation affects thermal tolerance. Subterranean organisms might be at an increased risk of decline in the face of global temperature rises, but robust data on the fauna is lacking, particularly at the molecular level. In this study we compare the heat shock response of two species of diving beetle in the genus *Paroster*: one surface-dwelling (*P. nigroadumbratus*), the other restricted to a single aquifer (*P. macrosturtensis*). *P. macrosturtensis* has been previously established as having a lower thermal tolerance compared to surface-dwelling relatives, but the genomic basis of this difference is unknown. By sequencing transcriptomes of experimentally heat-shocked individuals and performing differential expression analysis, we demonstrate both species can mount a heat shock response at high temperatures (35C), in agreement with past survival experiments. However, the genes involved in these responses differ between species, and far greater genes are differentially expressed in the surface species, which may explain its more robust response to heat stress. In contrast, the subterranean species significantly upregulated the heat shock protein gene *Hsp68* in the experimental setup under conditions it would likely encounter in nature (25C), suggesting it may be more sensitive to ambient stressors, e.g. handling. The results presented here contribute to an emerging narrative concerning weakened thermal tolerances in obligate subterranean organisms at the molecular level.

KEYWORDS: heat shock response, heat shock proteins, subterranean habitats, molecular evolution, transcriptomics, differential gene expression

1 | INTRODUCTION

The transition to an obligate subterranean lifestyle can cause massive shifts in a species' biology, from the acquisition of classic troglomorphies such as the elongation of appendages for sensing in an aphotic environment (Culver & Pipan, 2014) to changes in lesser-studied traits including circadian rhythm (Beale et al., 2016; Merritt & Clarke, 2011), reproductive biology (Walker & Rose, 1998), respiration (Jones *et al.* 2019) and chemosensation (Balart-García et al., 2021; J. Yang et al., 2016). These changes have been attributed to the stark difference between subterranean and surface (hereafter epigean) habitats. While epigean environments can vary immensely over both time and space, subterranean environments, such as cave systems, generally possess high environmental stability with respect to light levels, temperature, humidity, and nutrient availability (Badino, 2010). Animals adapted to these environments might therefore be particularly well suited for the assessment of responses to future climate change scenarios, particularly with respect to their thermal tolerances and responses to increasing temperatures. Indeed, such habitats have been labelled as undervalued "laboratories for studies of global change biology" (Mammola et al., 2019).

A near-universal response to temperature-induced stress across the tree of life–and therefore a method by which thermal tolerance can be gauged–is the heat shock response (hereafter HSR), which involves the synthesis of heat-shock proteins (HSPs). HSPs are expressed at base levels under non-stressful conditions and include those that are constitutively expressed (heat-shock cognates) - only induced when organisms are exposed to thermal extremes, during which they assist in stabilising and refolding proteins at risk of denaturation. An inducible HSR has been observed in almost all organisms studied to date, and the proteins involved in this response, as well as the response itself, are highly conserved among different domains of life (Jindal et al., 1989). An estimated 50 to 200 genes are involved in the HSR, the most significantly induced of which are HSPs (Richter et al., 2010).

71 However, there are exceptions to this rule: a lack of an inducible HSR has been documented
72 in a wide range of species, largely those that occupy very stable thermal environments such
73 as Antarctic marine habitats (Somero, 2020; Tomanek, 2010).
74 Knowledge of the HSR in organisms from thermally stable *subterranean* habitats,
75 including their associated inducible HSPs and at which temperatures this response might be
76 activated, is almost non-existent (e.g. Colson-Proch *et al.*, 2010). The vast majority of the
77 literature on this topic is also devoted to vertebrates, in stark contrast to their relatively small
78 contribution to the biodiversity of subterranean habitats, which is dominated by invertebrate
79 taxa (Gibert & Deharveng, 2002; Humphreys, 2012). Moreover, a better understanding of
80 responses to current climate change predictions for subterranean animals has been identified
81 as a fundamental question in subterranean biology given emerging conservation issues
82 associated with their respective ecosystems (Mammola et al., 2020). To address this
83 knowledge gap, here we make use of genomic data from Australian representatives of a
84 group of aquatic invertebrates containing both epigean and subterranean lineages.
85 The Yilgarn Craton in central Western Australia houses a diverse subterranean diving
86 beetle fauna belonging to two tribes, Bidessini and Hydroporini (Dytiscidae). While epigean
87 species can be found practically continent-wide, subterranean taxa are isolated in calcrete
88 aquifers (hereafter calcretes) associated with ancient palaeodrainage systems in the region.
89 These calcretes are completely devoid of light and animals contained within them are
90 assumed to have little to no access to air above the water's surface (Jones et al., 2019). Each
91 calcrete houses between one and three dytiscid species, but at the time of writing only around
92 a quarter of the ~200 known calcretes have been sampled (Cooper et al., 2002; Leijs et al.,
93 2012; Watts & Humphreys, 2009). Nonetheless, lineages in both tribes are known to have
94 made independent yet parallel, repeated transitions into underground habitats from epigean
95 ancestors during the late Miocene to early Pleistocene, likely in response to continental

96 aridification (Langille et al., 2021; Leijs et al., 2012; Leys et al., 2003; Toussaint et al., 2015).
97 In each case these transitions have involved the loss of eyes, pigment, and wings (Cooper *et*
98 *al.* 2002) as well as the gain of a remarkable ability to respire directly from water (Jones *et al.*
99 2019). Preliminary evidence suggests that subterranean members of these lineages are less
100 tolerant of thermal extremes compared to epigean relatives (Jones et al., 2021), but the
101 molecular mechanisms underlying such tolerances–and genomic changes associated with a
102 subterranean transition in these animals more broadly–remain unknown.
103 In the present study, we focus on two members of the Hydroporini: *Paroster*
104 *nigroadumbratus* (Clark), an epigean species endemic to South Australia, and the
105 subterranean *Paroster macrosturtensis* (Watts & Humphreys) found exclusively in a single
106 calcrete (hereafter the Sturt Meadows calcrete) in the Yilgarn region of Western Australia. As
107 subterranean dytiscids likely descended from only a handful of epigean lineages (Leijs et al.,
108 2012), meaningful comparisons can be made between these taxa despite their distributions
109 being geographically disjunct. A recent study showed that *P. macrosturtensis* has a reduced
110 upper thermal tolerance (or critical thermal maximum, CT_{max}) of 38.3ºC compared to other
111 epigean dytiscids (42–44.5ºC) (Jones et al., 2021), mirroring previous results for other cave
112 beetle species (Pallarés et al., 2020). These results also suggest *P. macrosturtensis* is unlikely
113 to reach its thermal critical maximum under current climate change predictions. However,
114 given the thermal stability in its environment it remains unknown as to whether *P.*
115 *macrosturtensis* might have a modified HSR compared to its epigean relatives. Building on
116 this previous work, here we present transcriptomic data from individuals of *P.*
117 *macrosturtensis* and *P. nigroadumbratus* previously subjected to varying degrees of heat
118 shock by Jones *et al*. (2021). In the present study we specifically aim 1) to generate a high-
119 quality, near-complete reference transcriptome for *P. nigroadumbratus* and 2) using this
120 dataset, characterise and compare the HSR of *P. nigroadumbratus* and *P. macrosturtensis*,

121 specifically with respect to which genes are differentially expressed and the conditions under
122 which this occurs.
123

124 # 2 | METHODS

125 ## 2.1 | Taxon sampling and experimental design

126 Beetle specimens were sourced from a previous study by Jones *et al*. (2021) that subjected
127 specimens to heat stress. Briefly, *P. macrosturtensis* samples (n = 11) were collected from the
128 Sturt Meadows calcrete in WA via existing mineral exploration boreholes. Epigean *P.*
129 *nigroadumbratus* specimens were collected from water bodies in the Mount Crawford region
130 of South Australia (n = 18). Individuals were kept by Jones *et al.* (2021) in constant
131 temperature cabinets at between 24 and 26ºC either in a 12:12h light-dark cycle (epigean
132 species) or in complete darkness (subterranean species) for at least seven days prior to
133 subsequent experimentation to minimise the effects of variable thermal history and stress
134 during capture and transport. Beetles were then either retained in this constant temperature
135 environment and euthanized in liquid N_2 (control treatment) or moved to a temperature-
136 controlled water bath containing pure reverse osmosis (RO) water (for epigean species) or
137 RO water with a salinity level mimicking calcrete microhabitats (for subterranean species).
138 Beetles were kept at 25ºC in this environment for 4.5 hours to determine if they could survive
139 the experimental setup (25ºC treatment) or allowed to acclimate to 25ºC for 1 hour before the
140 temperature was increased by 0.05ºC every minute until it reached 35ºC (35ºC treatment). In
141 all cases individuals were euthanized in liquid N_2 immediately after this time period.
142

143 ## 2.2 | cDNA synthesis and sequencing

144 RNA extractions of control and heat-treated individuals were performed using single whole
145 bodies per treatment. Extractions were performed using an AllPrep DNA/RNA Micro kit

146 (Qiagen, Chadstone, VIC), initially homogenizing in lysis buffer containing β-
147 mercaptoethanol plus a 5 mm steel bead on a Mixer Mill (Retsch, Haan, Germany). Lysates
148 were processed following the manufacturer's instructions and RNA was eluted from the
149 RNeasy MinElute spin column in 25 µl RNAse-free water. Concentration of the RNA extract
150 was determined using a Quantus Flourometer (Promega, Madison, Wisconsin, US) and RNA
151 integrity was analysed on a TapeStation (Agilent Technologies, Santa Clara, California, US).
152 Library preparation was performed using a Nugen Universal Plus mRNA-Seq Library
153 Preparation Kit (Tecan, Männedorf Switzerland), following manufacturer's instructions. All
154 the barcoded cDNA samples were pooled and 100 bp paired-end sequencing was outsourced
155 to the Australian Genome Research Facility (AGRF) on an Illumina NovaSeq 6000 S4 lane.
156 The reference transcriptome for *P. nigroadumbratus* presented here was derived from
157 previously obtained raw RNA-seq data (Tierney et al., 2015).
158

159 **2.3 | Quality control of sequence data**

160 Trim Galore v.0.4.1 (bioinformatics.babraham.ac.uk/projects/trim_galore), a wrapper that
161 implements Cutadapt (Martin, 2011) and FastQC
162 (bioinformatics.babraham.ac.uk/projects/fastqc), was used for quality control of our raw
163 reads and the removal of residual sequencing adapters, with default settings for paired
164 Illumina reads. This protocol was applied to both the raw reads from control/heat-treated
165 samples and those intended for reference transcriptome assembly.
166

167 **2.4 | Assembly of *Paroster nigroadumbratus* reference transcriptome**

168 Following quality control and adapter removal, Trinity v.2.5.1 (Grabherr et al., 2011) was
169 used for *de novo* transcriptome assembly of our raw reads using default settings. Bowtie
170 v.1.1.2 (Langmead et al., 2009) was used to examine RNA-Seq read representation in the

171 assembly by aligning raw reads to the assembled transcriptome. Reference transcriptome
172 completeness was validated using the gVolante implementation of BUSCO v.5 (Nishimura et
173 al., 2017; Simão et al., 2015) with default settings, searching against the Arthropoda
174 database.

175 Functional annotation of the transcriptome was performed using TransDecoder v.5.5.0
176 (Haas et al., 2013) to predict coding regions in transcripts, Trinotate v.3.2.1 (Grabherr et al.,
177 2011) and SQLite (http://sqlite.org/index.html) for database integration, and BLAST v.2.7.1
178 (Camacho et al., 2009) and HMMER v.3.2 (http://hmmer.org) for homology searches and the
179 identification of protein domains. BLASTX and BLASTP searches were conducted against
180 the Swiss-Prot database v.2021_02 (The UniProt Consortium, 2021) for Trinity transcripts
181 and TransDecoder-predicted proteins, respectively. These searches were performed with an e-
182 value of 1-e6 to capture more distantly matching proteins and *-max_hsps* and -
183 *max_target_seqs* both set to 1 to limit our results to the best hit per query by e-value. While
184 the use of the latter parameter has been subject to scrutiny as top hits are selected based on
185 sequence order in the database in the case of equivalent matches, this behaviour appears to
186 occur in datasets with an extremely high proportion of gaps (Madden et al., 2019; Shah et al.,
187 2019), which does not apply to the present study. The Pfam database v.34.0 (Mistry et al.,
188 2021) was used in HMMER searches to identify protein domains with default settings.

189 Finally, we subsetted the ORFs inferred by TransDecoder to only include the longest
190 isoform per gene. These transcripts were used in downstream analyses to reduce redundancy
191 and allowed us to perform read quantification and differential expression analysis at the
192 "gene" level as opposed to that of isoforms following Balart-García *et al*. (2021).
193

194 **2.5 | Differential expression analysis and Gene Ontology enrichment**
195 **analysis**

196 Salmon v.1.1.0 (Patro et al., 2017) was used to index our annotated *P. nigroadumbratus*
197 reference transcriptome and quasi-map our raw reads from control/heat-treated samples
198 against it with an automatic inference of library type and 100 bootstrap replicates. Reads
199 were normalised with the trimmed mean of M-values normalization method (TMM). TMM
200 read counts for each replicate were visualised using principal components analysis consisting
201 of a total of 13 *P. nigroadumbratus* replicates and 11 for *P. macrosturtensis* in our
202 downstream differential expression analysis (Figure S1).

203 The Trinity helper scripts for edgeR v.3.32.1 (Robinson et al., 2010) were used for
204 differential expression analyses and enrichment of Gene Ontology (hereafter GO) terms
205 based on annotations from the *P. nigroadumbratus* reference transcriptome. Abundance
206 estimates from Salmon were first transformed for use in edgeR prior to running differential
207 expression analysis with default settings (*abundance_estimates_to_matrix.pl,*
208 *run_DE_analysis.pl*). Differential expression analysis results were then analysed with a *p*-
209 value cut-off for FDR of 0.05 and a fold-change of 1. This analysis included the
210 enrichment/depletion of GO terms by supplying Trinity gene lengths and GO terms for each
211 annotation (Young et al., 2010) and compared 25ºC and 35ºC treatments against individuals
212 of the same species in the control treatment.

213 Significantly up- and downregulated genes shared between different treatments were
214 identified using a custom Venn diagram webtool
215 (bioinformatics.psb.ugent.be/webtools/Venn) and visualised with UpSetR v.1.4.0 (Conway et
216 al., 2017). Enrichment of GO terms was visualised with REVIGO (Supek et al., 2011), which
217 summarises terms by clustering them and retrieving a meaningful subset using similarity
218 measures. Enriched GO terms were supplied alongside FDR values associated with
219 enrichment/depletion and the REVIGO analysis was performed using an allowed similarity of

220 0.9. Resulting tree maps were produced with values set to abs(log10(*p*-value)) to ensure plots
221 reflected the significance of enrichment of each term.
222

223 2.6 | Phylogenetic analysis of putative heat shock proteins

224 Genes annotated as putatively encoding heat shock proteins consisted of members of three of
225 the four insect families of HSP, including small heat shock proteins (sHSP), HSP70, and
226 HSP90. Transcripts were extracted on the basis of annotations containing the terms *heat*
227 *shock*, *HSC*, *HSP*, or *chaperone*. To ensure the inclusion of as many genes as possible for
228 which annotation might have been incomplete (e.g. certain HSP chaperones), we also
229 included transcripts putatively involved in the HSR that were differentially expressed in our
230 dataset yet not captured in the above search. The resulting candidate set of 38 transcripts was
231 checked against the UniProt database to confirm the function of the proteins they encoded,
232 and the identities of transcripts were then validated using BLASTN searches restricted to the
233 Coleoptera against the NCBI nr database. These transcripts were used as a reference to filter
234 raw reads from each *P. macrosturtensis* 35ºC replicate using BWA v.0.7.17 (H. Li & Durbin,
235 2009) and SAMtools v.1.9 (H. Li et al., 2009). Filtered *P. macrosturtensis* reads (excluding
236 singletons) were then pooled and mapped against reference transcripts using the Read
237 Mapper tool in Geneious Prime (v.2021.2.2, geneious.com) with Low Sensitivity presets.
238 Consensus sequences for *P. macrosturtensis* were produced from these alignments with a 0%
239 (majority) threshold.
240 Coleopteran orthologs of the above genes were retrieved from OrthoDB v.10.1
241 (Kriventseva et al., 2019) with LOC identifiers of the top BLASTN hit as queries. mRNA
242 transcripts of protein sequences in question were obtained from GenBank, representing
243 sequences from *Aethina tumida* Murray (Polyphaga: Cucujoidea), *Agrilus planipennis*
244 Fairmaire (Polyphaga: Buprestoidea), *Anoplophora glabripennis* (Motschulsky) (Polyphaga:

245 Chrysomeloidea), *Dendroctonus ponderosae* (Hopkins) (Phytophaga: Curculionoidea),
246 *Leptinotarsa decemlineata* Say (Polyphaga: Chrysomeloidea), *Nicrophorus vespilloides*
247 Herbst (Polyphaga: Staphylinoidea), *Onthophagus taurus* (Schreber) (Polyphaga:
248 Scarabaeoidea), and *Tribolium castaneum* (Herbst) (Polyphaga: Tenebrionoidea) alongside
249 our two species of interest (summarised in Table S1). Alignments were computed using
250 MUSCLE v.3.8.425 (Edgar, 2004), checked by eye to ensure all transcripts were in frame,
251 and trimmed to exclude portions of GenBank sequences outside of open reading frames.
252 Partitions were inferred using PartitionFinder v.2.1.1 (Lanfear et al., 2017), with a resulting
253 partitioning scheme by codon position. Phylogenetic analysis of HSPs was performed using
254 maximum likelihood with RAxML v.8.2.12 (Stamatakis, 2014) via the CIPRES Science
255 Gateway (Miller et al., 2010) with default settings and 1000 bootstrap replicates.
256

257 # 3 | RESULTS

258 ## 3.1 | A high-quality reference transcriptome for *Paroster nigroadumbratus*
259 ## enables the characterisation of genes upregulated during the heat shock
260 ## response

261 Here we present a high-quality, near-complete transcriptome for the epigean beetle *P.*
262 *nigroadumbratus*. This dataset consisted of 75,045,266 paired-end reads (72,266,264
263 following quality control measures), 84% of which were incorporated into 134,246 *de novo*
264 assembled transcripts representing 60,683 unique Trinity "genes" and 41,979 predicted
265 ORFs. According to assessment in BUSCO, this transcriptome was 87.66% complete with
266 respect to core arthropod genes and 96.74% complete when considering partial genes. Of
267 these, 47,810 transcripts were functionally annotated using the Trinotate pipeline. Subsetting
268 our predicted peptide dataset to include only the longest isoforms per putative locus, allowing

269 us a proxy with which to perform our downstream analyses at the gene level, resulted in
270 14,897 predicted ORFs (with 11,609, or ~77%, having some level of annotation).
271

272 3.2 | Differential expression, GO enrichment analysis reveal distinct 273 expression profiles associated with the heat shock response

274 Differential expression analysis in edgeR identified a total of 802 differentially expressed
275 (DE) genes in the epigean *P. nigroadumbratus* samples and 153 in the subterranean *P.*
276 *macrosturtensis*, with the two species exhibiting complex and markedly different expression
277 profiles (Figures 1, S2, S3). Different transcripts variants of the same putative homolog were
278 often differentially expressed across temperature treatments; these are indicated in Figure 1 as
279 bracketed numbers after gene names to avoid confusion. *P. macrosturtensis* upregulated
280 *Hsp68* at both 25ºC and 35ºC, a transcript only upregulated at 35ºC in *P. nigroadumbratus*.
281 No differentially expressed heat shock proteins or cochaperones were shared between 25ºC
282 and 35ºC treatments exclusively in one species, nor were any differentially expressed
283 exclusively at 25ºC in *P. macrosturtensis* and not shared by any other treatments. At 35ºC,
284 both species upregulated the HSP genes *Hsp83* and *Hsc70-4* and the cochaperones *Samui*,
285 *BAG2*, *DnaJ*, *AHSA1, and Unc45a*. Gene expression profiles unique to each species
286 involved:1) the downregulation of two distinct transcripts each of *l(2)efl* and *Hsp68* in *P.*
287 *nigroadumbratus* at 25 ºC; 2) the downregulation of the *BAG6* cochaperone and the
288 upregulation of *Hsc70-3* and *l(2)efl* and *Hsp68* in *P. nigroadumbratus* at 35ºC; and finally 3)
289 the upregulation of *Hsc70-2*, *Hsp68* and sHSPs *l(2)efl* and *HspB1* in *P. macrosturtensis* at
290 35ºC. Interestingly, *Hsp68* transcripts upregulated in *P. macrosturtensis* at 25ºC were the
291 same as those downregulated in *P. nigroadumbratus* under the same conditions. Similarly,
292 *l(2)efl* and the aforementioned *Hsp68* transcripts downregulated at 25ºC were also
293 upregulated at 35ºC in *P. nigroadumbratus*. Other annotated genes thought to be involved in

294 the HSR, such as *Hsc70-2*, *Hsc70-5*, *TRAP1*, and *Hsp90b1*, were not differentially expressed
295 in either species. A full list of differentially expressed genes shared between (or unique to)
296 treatments is available in Table S2.
297 Gene Ontology enrichment analysis enables the visualisation and collapse of similar
298 GO terms associated with differentially expressed genes. We did not observe any consistent
299 depletion of GO terms associated with downregulated genes, but enriched terms were
300 reflective of our differential expression results above (Figure 2). In both species, terms
301 associated with the HSR were enriched at 35ºC (e.g. ATPase, chaperone binding, and
302 unfolded protein binding). At 25ºC, ADP binding was also enriched for *P. macrosturtensis*
303 due to the upregulation of *Hsp68* at that treatment.
304

305 **3.3 | Phylogenetic analyses**

306 Our HSP nucleotide alignments consisted of 67 sequences across 3,669bp (HSP70 family),
307 29 sequences across 3,741bp (HSP90 family) and 23 sequences across 867bp (sHSP family).
308 Three major clades were recovered in our phylogeny with strong node support (Figure 3). All
309 sequences from the *Paroster* species examined here were recovered as nested within these
310 clades, confirming the orthology of annotated HSPs, with the exception of a transcript present
311 in both species and inferred as sister to the *Hsp68*+*Hsc70-2* clades. This transcript was not
312 differentially expressed in response to heat shock in our dataset.
313

314 **4 | DISCUSSION**

315 Here, we have comprehensively characterised the heat-shock response at the molecular level
316 in the subterranean diving beetle *Paroster macrosturtensis* and one of its surface-dwelling
317 relatives. Using a near-complete reference transcriptome for *P. nigroadumbratus*–the first
318 such dataset for a member of the Hydroporinae–we performed differential expression and GO

319 enrichment analysis to explore genes putatively involved in the HSR. Our results demonstrate
320 that both the epigean *P. nigroadumbratus* and subterranean *P. macrosturtensis* have an
321 inducible HSR, mirroring previous survival experiments for the genus (Jones et al., 2021).
322 Putative orthologs of HSP cochaperones, sHSP, HSP70, and HSP90 genes were accounted
323 for in our differential expression analysis. However, the conditions under which this response
324 is activated differs between species, and *P. macrosturtensis* notably differentially expressed
325 less than 20% of the number of genes in response to heat stress compared to *P.*
326 *nigroadumbratus*.
327

328 **4.1 | Heat shock-induced expression profiles of *Paroster* species**

329 HSPs identified as differentially expressed in this study support past results from beetle
330 species and other insects more broadly. A major trend from our results concerned the
331 upregulation of HSP70 genes, particularly *Hsp68*; HSP70s are highly expressed in response
332 to heat shock in the Colorado potato beetle (Yocum, 2001), red flour beetle (Chen et al.,
333 2020), and the cave beetle *Neobathyscia* (Bernabò et al., 2011). These genes are upregulated
334 in concert with sHSPs and HSP90 genes in the rice weevil (Yuan et al., 2014) and Japanese
335 sawyer beetle (Cai et al., 2017) under the same conditions. HSP70s and sHSPs are also
336 known to be upregulated in response to heat shock in the cigarette beetle (M.-Y. Li et al.,
337 2021; W.-J. Yang et al., 2019).
338 In addition to the heat shock proteins, we also observed the upregulation of the heat
339 shock cognate *Hsc70-4* and cochaperones *DnaJ*, *Samui*, *BAG2*, and *AHSA1* in response to
340 heat shock in *Paroster*. To our knowledge there has been no documentation of a coleopteran
341 heat shock cognate being upregulated in response to increasing temperature, though evidence
342 exists for the parasitic wasp *Pteromalus*, in which *hsc70* is also induced by heavy metal
343 poisoning and starvation in addition to thermal extremes (H. Wang et al., 2012). Heat shock

344 cognates are also upregulated during diapause in silkworm eggs (Sasibhushan et al., 2012)
345 and young bumble bee queens (Kim et al., 2008), potentially playing a cryoprotective role in
346 these species. Cochaperones are less well characterised in insects, but evidence for their
347 upregulation in response to heat shock has been documented for *DnaJ* in hemipterans (Huang
348 & Kang, 2007; Hull et al., 2013) and BAG-family members, such as *Samui,* in ants (Tonione
349 et al., 2020). Finally, we also observed the downregulation of multiple heat shock proteins in
350 both species. In *P. nigroadumbratus* this was restricted to *BAG6* (a cochaperone) at 35ºC, and
351 *l(2)efl* and *Hsp68* at 25ºC. Both sHSPs and HSP70s have been documented as being
352 downregulated during periods of heat stress in other insects, e.g. in silk moths (Sakano et al.,
353 2006).
354

355 **4.2 | The subterranean species *Paroster macrosturtensis* retains a heat**
356 **shock response**

357 The dataset presented here adds to a growing body of knowledge concerning the HSR in
358 organisms that inhabit thermally stable environments. Central to discourse on this topic is the
359 climatic variability hypothesis, which posits that the thermal tolerance of a taxon is positively
360 correlated with its temperature ranges encountered in nature (Stevens, 1989). This hypothesis
361 implies species from extremely stable thermal environments can no longer tolerate
362 temperature extremes, and has been demonstrated in a wide variety of organisms that have
363 either lost or possess a reduced HSR, such as cnidarians (Brennecke et al., 1998), limpets
364 (Sanders et al., 1991), amphipods and sea stars (Clark et al., 2008), and midges (Rinehart et
365 al., 2006). However, there are exceptions to this rule, with groundwater-dwelling organisms
366 such as those examined here displaying an inducible HSR in response to conditions far
367 warmer than they would encounter in nature (Colson-Proch et al., 2010; Mermillod-Blondin
368 et al., 2013). The HSR of these species, as well as *P. macrosturtensis*, might be retained at

369 such high temperatures for a variety of reasons, including the fact that HSPs play a
370 pleiotropic role in responding to other forms of stress and/or these species have not occupied
371 their respective environments for a sufficient length of time in evolutionary terms for their
372 HSR to be lost, e.g. via adaptive processes or a relaxation of purifying selection (Jones et al.,
373 2021). The latter hypothesis is plausible for *Paroster* as *P. macrosturtensis* is also known to
374 have retained the ability to detect light despite inhabiting an aphotic environment for over ~3
375 million years (Langille et al., 2019).

376 The present study not only demonstrates that *P. macrosturtensis* has retained an
377 inducible HSR, but that its HSR appears to differ markedly from its epigean relative *P.*
378 *nigroadumbratus* at the molecular level. While *P. macrosturtensis* is endemic to the Sturt
379 Meadows calcrete in which groundwater temperatures are relatively narrow and do not rise
380 above approximately 28ºC, individuals can survive at up to 38ºC before experiencing severe
381 locomotive impairment (Jones et al., 2021). The retention of a HSR in *P. macrosturtensis*
382 supports these physiological findings. However, almost half (40%) of the *P. macrosturtensis*
383 cohort did not survive 24 hours after heat shock compared to zero deaths with respect to *P.*
384 *nigroadumbratus* individuals. Given the reduced thermal tolerance of *P. macrosturtensis*, it
385 remains to be seen if the species could tolerate such extremes in the long term. Indeed, even
386 cave beetles considered stenothermal–those that are only capable of surviving within an
387 extremely narrow temperature range–have retained the HSR (Pallarés et al., 2020), but
388 nonetheless cannot survive at extreme temperatures for long periods (>7 days) compared to
389 epigean relatives (Bernabò et al., 2011; Rizzo et al., 2015).

390 Our molecular data reflects the previously documented reduced thermal tolerance of
391 subterranean dytiscids such as *P. macrosturtensis*. In keeping with these findings, *P.*
392 *macrosturtensis* exhibited significant upregulation of a transcript of *Hsp68* at 25ºC that was
393 also enriched in all 35ºC treatments in response to temperature extremes. These results do not

394 necessarily imply *Hsp68* was upregulated in response to heat stress at 25ºC, as HSPs are
395 known to be involved in responding to a wide range of stressors (King & MacRae, 2015;
396 Sang et al., 2012; Sørensen et al., 2003) 25ºC is within the range of temperatures *P.*
397 *macrosturtensis* might encounter in nature (average 23ºC–26.2ºC) (Jones et al., 2021), and
398 this was the same temperature (± 1ºC) as the control treatment of Jones *et al*. (2021). On the
399 other hand, the upregulated *Hsp68* transcript variant was also differentially expressed in the
400 two 35ºC treatments in both species, potentially suggesting a link to heat stress.
401 The above could indicate *P. macrosturtensis* 1) did experience some initial degree of heat
402 shock response in the control treatment and this was further exacerbated by the addition of
403 the experimental setup in the 25ºC treatment, or 2) the response was triggered by stress
404 induced by being moved to designated 25ºC vials, given *P. nigroadumbratus* also exhibits
405 differential expression of HSPs at 25ºC (though the downregulation of transcripts therein are
406 not shared with other treatments) (Figure 1). The former scenario would reflect the reduced
407 thermal tolerance of *P. macrosturtensis*, which is also supported by the species differentially
408 expressing a far lower number of genes in response to higher temperatures compared to *P.*
409 *nigroadumbratus*. Similarly reduced numbers of differentially expressed genes have also
410 been associated with lower thermal tolerances in other organisms such as fish (Narum &
411 Campbell, 2015), lizards (Akashi et al., 2016), rotifers (Paraskevopoulou et al., 2020), red
412 algae (W. Wang et al., 2018), and plants (Jin et al., 2020; Sun et al., 2021), though we note
413 the inverse (or alternatively, no clear pattern) has been observed in a number of cases,
414 potentially reflecting lower levels of stress as opposed to an inability to mount a HSR
415 (DeBiasse & Kelly, 2016; Perez et al., 2021).
416 Acquiring data such as these is not only necessary to better understand the HSR in
417 subterranean animals in the face of climate change, but also to inform future conservation
418 decisions. The latter is of particular relevance to members of subterranean *Paroster* as the

419 groundwater in the palaeovalleys containing their calcrete habitats, as well as the calcretes
420 themselves, are heavily utilised for mining activities. As short-range endemics to the extreme,
421 these species are not only at risk of habitat degradation via the direct intersection of shallow
422 aquifers with mining activities, but also through the drawdown of groundwater beneath
423 calcretes at greater depths (Hose et al., 2015). Dytiscids (and stygofauna more broadly) in
424 these regions therefore not only face population declines or extinctions due to the increasing
425 threat of climate change, but also potentially co-occurring stressors from mining operations
426 such as changes in temperature, quality, and depth of groundwater. Accordingly, the calcretes
427 these species inhabit are considered a top conservation priority for protection by the
428 Commonwealth Scientific and Industrial Research Organisation (Healy, 2015). Such threats
429 are of particular concern for obligate stygofaunal species given the data presented by Jones *et*
430 *al.* (2021) and, more broadly, the fact that the thermal critical maximum of subterranean
431 beetle species is known to decrease with the degree of subterranean specialisation (Pallarés et
432 al., 2020). An increased knowledge of the assumed fragility of Australian subterranean
433 dytiscids in the face of these stressors is therefore crucial for informing future conservation
434 decisions.

435 Overall, the findings presented here demonstrate the reduced thermal tolerance of the
436 subterranean species *P. macrosturtensis* compared to its epigean relatives is reflected in, and
437 further clarified by, transcriptomic data. While our data are supported by past physiological
438 evidence that demonstrated *P. macrosturtensis* could survive at high temperatures, albeit not
439 to the limits of epigean species (Jones et al., 2021), the present study adds a new layer to this
440 narrative. *P. macrosturtensis* might possess increased mortality in the face of high
441 temperatures and a lower maximum temperature it can tolerate because 1) the species
442 differentially expresses far fewer genes in response to heat shock compared to the epigean
443 relative *P. nigroadumbratus*, suggesting it is unable to mount a robust HSR, and 2) this

444 response appears to be triggered at lower temperatures and/or in response to ambient
445 stressors, potentially subjecting individuals to stress for longer periods of time. Ultimately, it
446 remains unknown as to how *P. macrosturtensis* might respond to temperature extremes in the
447 long term, such as under a predicted increase of up to ~5ºC by 2090 in central Western
448 Australia (Healy, 2015). Nonetheless, the apparently weaker HSR we have characterised for
449 the species implies these short range endemics may not be able to thrive in such conditions,
450 especially given the threat of more immediate human-induced stressors such as mining
451 activities.
452

453 **5 | CONCLUSIONS**

454 The present study contrasted differentially expressed genes involved in the heat shock
455 response (HSR) of *Paroster nigroadumbratus* and *P. macrosturtensis*, epigean and
456 subterranean lineages of hydroporine diving beetles endemic to Australia previously
457 subjected to heat shock by Jones *et al.* (2021). Our results have comprehensively
458 characterised the genes involved in the HSR in these species and demonstrate that the
459 subterranean *P. macrosturtensis* retains a HSR at the molecular level, in agreement with the
460 physiological data of Jones *et al.* (2021) detailing that the species can survive temperatures
461 far above what it would encounter in nature. However, we also show that *P. macrosturtensis*
462 is potentially less equipped at the molecular level to respond to higher temperatures, with the
463 species differentially expressing less than a fifth of the number of genes in response to heat
464 shock in *P. nigroadumbratus* at 35ºC. Together, these results support the idea that *P.*
465 *macrosturtensis* has lost some degree of tolerance to temperature extremes compared to
466 epigean species such as *P. nigroadumbratus*, in agreement with the climatic variability
467 hypothesis. Ultimately, sampling of additional subterranean dytiscid species in the Yilgarn

region will enable a more holistic assessment of the thermal tolerances of this unique natural experiment at the molecular level.

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783 **DATA ACCESSIBILITY STATEMENT**

784 All raw RNA-seq data for *Paroster macrosturtensis* and *P. nigroadumbratus* used in differential
785 expression analyses in this study are available under BioProject ID PRJNA783065 via the Sequence
786 Read Archive. HSP transcripts assembled from raw *P. macrosturtensis* RNA-seq data and used in
787 phylogenetic analysis are available via GenBank under accession numbers OL803873-OL803888
788 (summarised in Table S1). The reference transcriptome of *P. nigroadumbratus* (including all isoforms)
789 and the annotated, subsetted dataset (only including longest isoform per Trinity "gene") are available via
790 FigShare (DOI: 10.25909/17169191).

791

792 **AUTHOR CONTRIBUTIONS**

793 PGBH and SJBC conceived the study. PGBH analysed the data and wrote the manuscript. TB and CSPF
794 provided guidance regarding bioinformatic analyses. ST performed molecular laboratory work. TMB,
795 KJ, WFH, and ADA helped to draft the manuscript. All authors edited the manuscript and approved the
796 final draft.

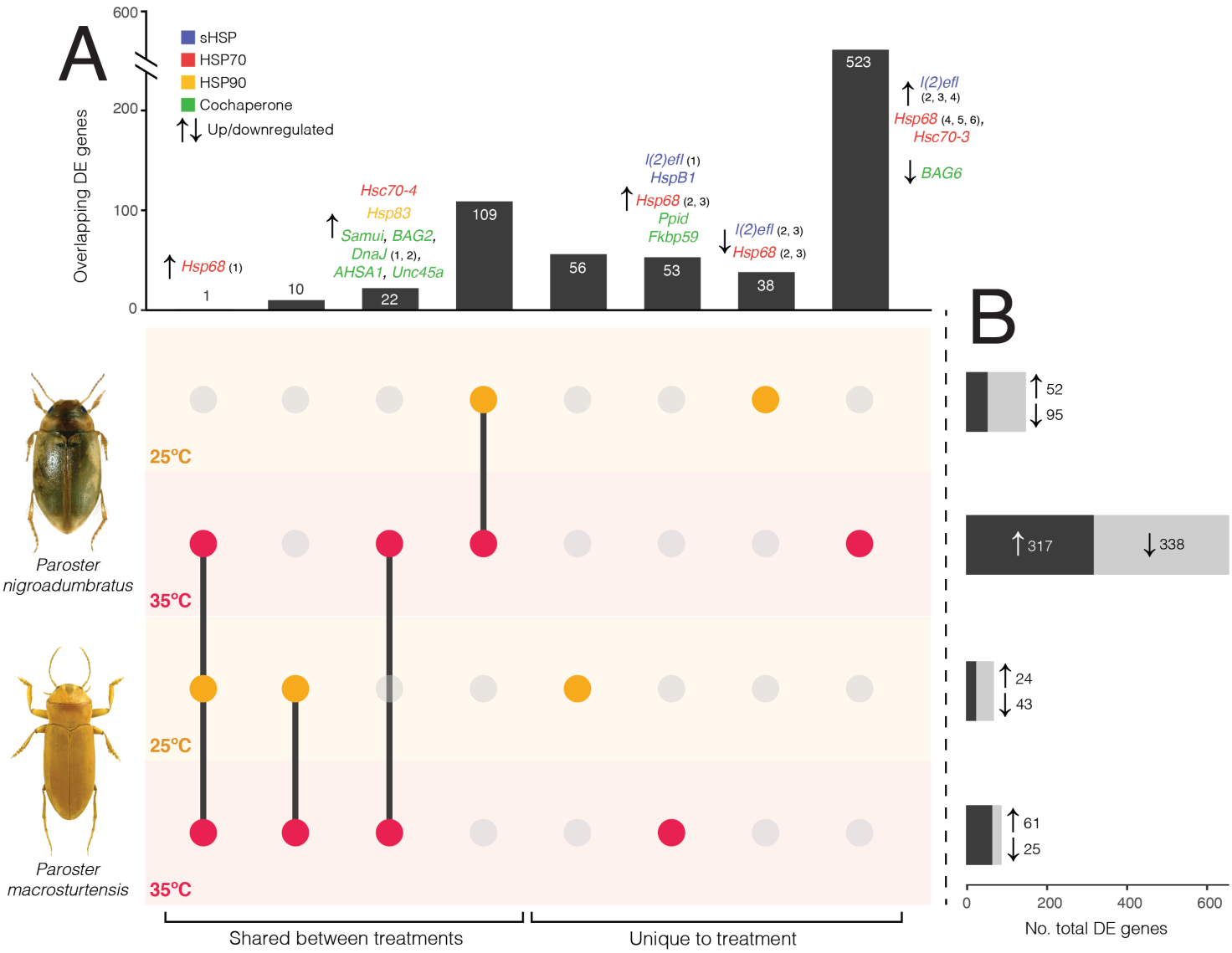

FIGURE 1 Differentially expressed (DE) genes in *Paroster nigroadumbratus*, a surface-dwelling species, and *P. macrosturtensis*, a subterranean relative, in response to 25ºC and 35ºC treatments *vs.* control treatment (25ºC without the experimental setup) by Jones *et al*. (2021). A) DE genes shared (denoted by black lines linking circles) or unique to (unlinked circles) each temperature treatment per species. The number of shared or unique DE genes among treatments is shown in the bar graph above, including names of DE HSPs and/or HSP cochaperones. Genes are shown as either upregulated or downregulated, denoted with arrows. Bracketed numbers in the bar graph indicate different transcripts of the same gene. B) total DE genes for each temperature treatment *vs*. control, irrespective of overlap with other treatments. Photographs by Chris Watts and Howard Hamon.

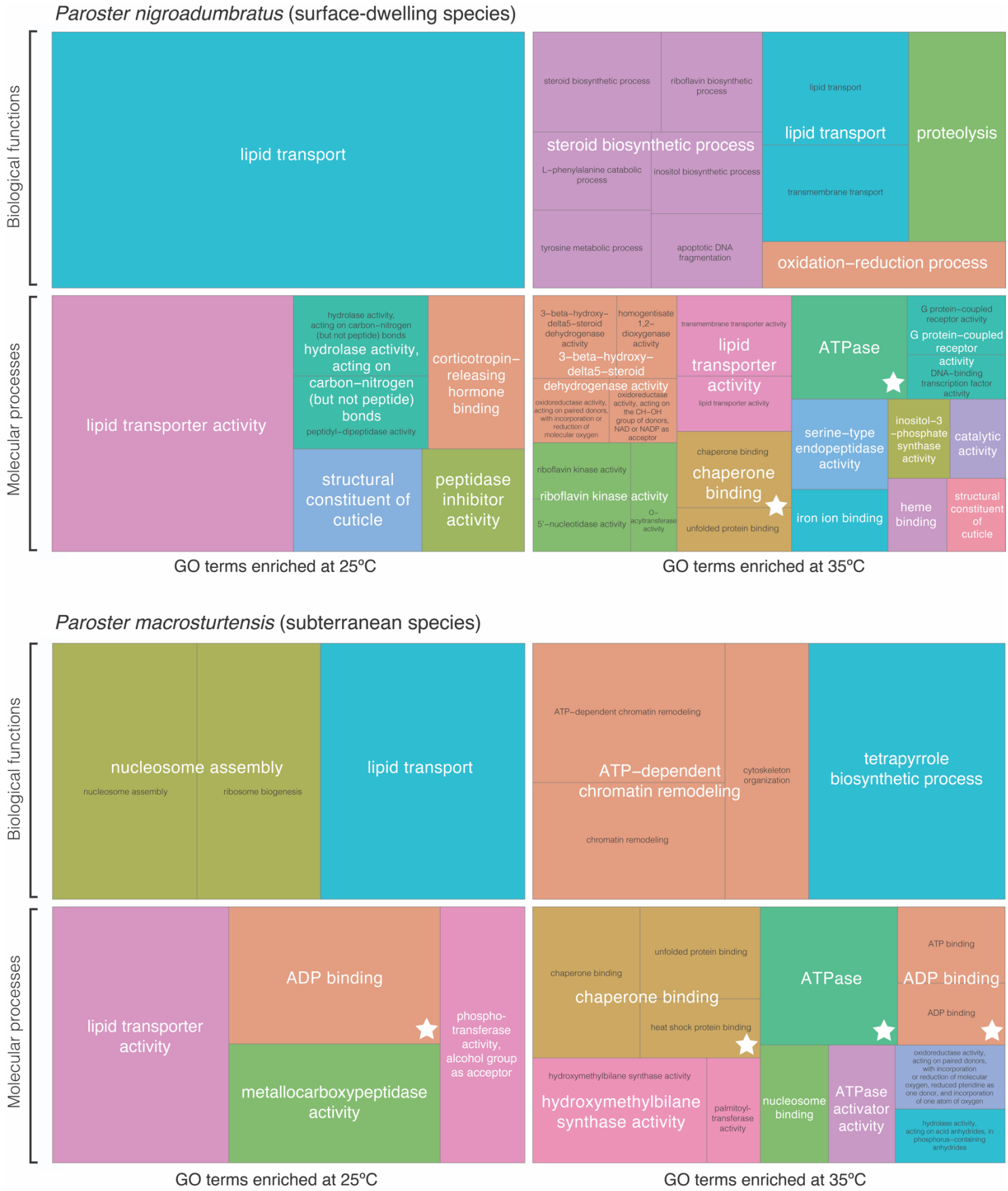

FIGURE 2 REVIGO treemap showing Gene Ontology (GO) terms associated with upregulated genes at 25ºC and 35ºC compared to the control treatment. The size of squares in the figure is proportional to the *p*-value associated with differential expression of respective genes. Similar GO terms share a colour and are represented in white text by the largest square per group. Terms associated with heat shock shown here include ADP binding, chaperone binding, and ATPase, indicated by a star.

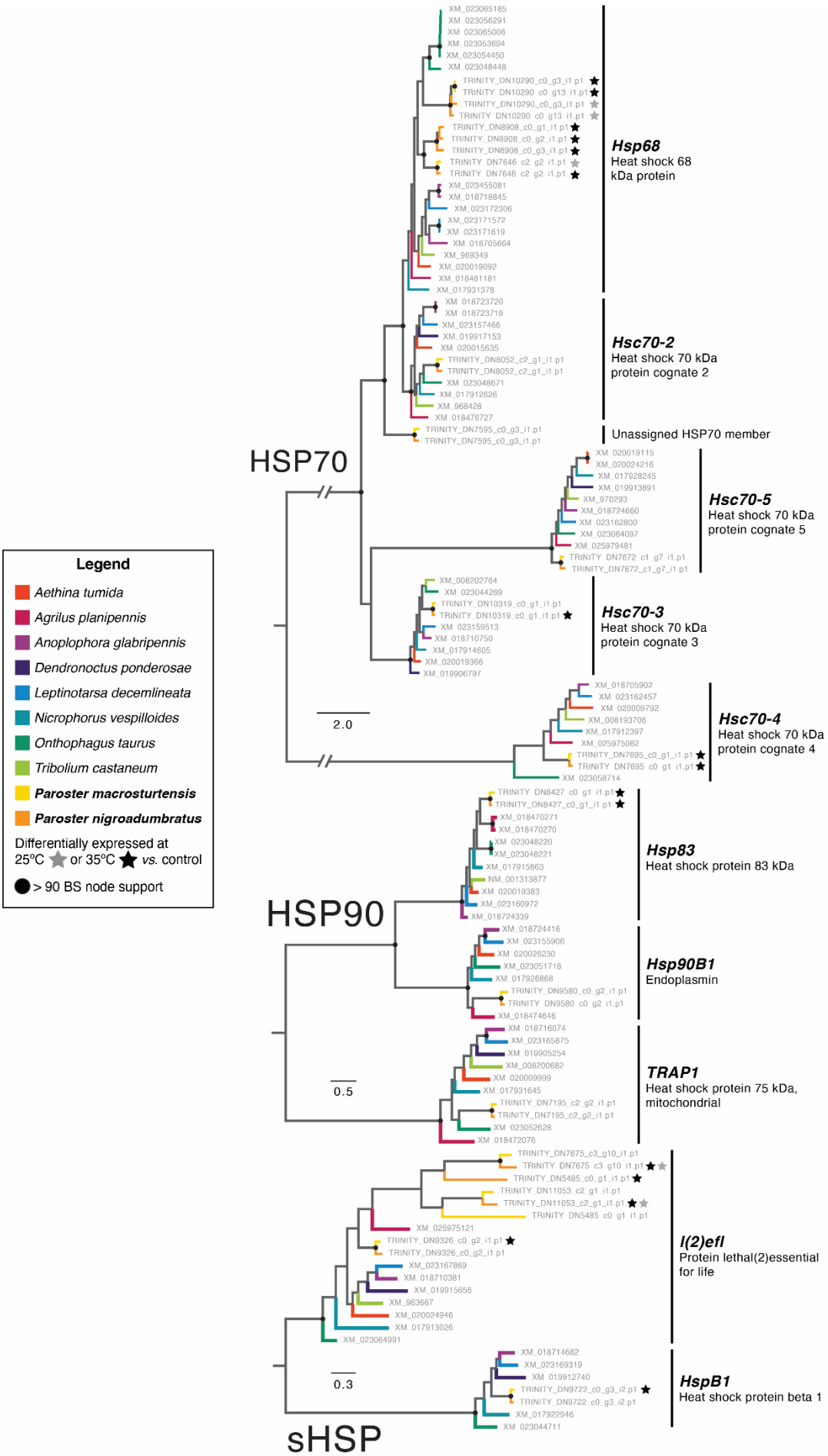

FIGURE 3 Phylogeny of heat shock transcripts inferred in RAxML to validate the identity of putative HSP orthologs in *Paroster* species. Transcript variants are represented by branches of the same colour within a clade. Scale bar is in substitutions/site; BS = bootstrap node support. Tip labels show transcript names/GenBank accession numbers. Tip names for *P. macrosturtensis* are shared with the reference *P. nigroadumbratus* transcript that reads were assembled against. Differentially expressed transcripts under a certain condition are with respect to the control.