Differential transcriptomic responses to heat stress in surface and subterranean diving beetles

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Abstract

Subterranean habitats are generally very stable environments, and as such evolutionary transitions of organisms from surface to subterranean lifestyles may cause considerable shifts in physiology, particularly with respect to thermal tolerance. In this study we compared responses to heat shock at the molecular level in a geographically widespread, surface-dwelling water beetle to a congeneric subterranean species restricted to a single aquifer (Dytiscidae: Hydroporinae). The obligate subterranean beetle *Paroster macrosturtensis* is known to have a lower thermal tolerance compared to surface lineages (CT_{max} 38ºC *cf.* 42-46 ºC), but the genetic basis of this physiological difference has not been characterized. We experimentally manipulated the thermal environment of 29 individuals to demonstrate that both species can mount a heat shock response at high temperatures (35ºC), as determined by comparative transcriptomics. However, genes involved in these responses differ between species and a far greater number were differentially expressed in the surface taxon, suggesting it can mount a more robust heat shock response; these data may underpin its higher thermal tolerance compared to subterranean relatives. In contrast, the subterranean species examined not only differentially expressed fewer genes in response to increasing temperatures, but also in the presence of the experimental setup employed here alone. Our results suggest *P. macrosturtensis* may be comparatively poorly equipped to respond to both thermally induced stress and environmental disturbances more broadly. The molecular findings presented here have conservation implications for *P. macrosturtensis* and contribute to a growing 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 an organism's biology (1), from the acquisition of classic troglomorphies such as the elongation of appendages for sensing in an aphotic environment (2), to changes in lesser-studied traits including circadian rhythm (3,4), reproductive biology (5), respiration (6), the number of larval instars (7,8) and chemosensation (9,10). 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 (11–13). 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 natural laboratories for biological studies of global change (14).

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 (hereafter HSPs). HSPs include those proteins that are expressed constitutively under non-stressful conditions, called heat shock cognates, or those 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 (15). An estimated 50 to 200 genes are involved in the HSR, the most significantly induced of which are HSPs (16). However, there are exceptions to this rule: a lack of an inducible HSR has been documented in a wide range of species, largely those that occupy very stable thermal environments such as Antarctic marine habitats (17,18).

71 Knowledge of the HSR in organisms from thermally stable *subterranean* habitats, including
72 their associated inducible HSPs and at which temperatures this response might be activated, is
73 scarce, with only a few studies devoted to invertebrate taxa (14,19–25). However, invertebrates
74 overwhelmingly contribute to the biodiversity of subterranean habitats compared to vertebrates
75 (26,27). The bulk of existing studies on the thermal tolerance of subterranean invertebrates suggests
76 such taxa can withstand temperatures above those they would encounter in nature and suggest they
77 have not lost the HSR. However, only a small number of these studies have examined the heat shock
78 response directly (28) and tend to focus on thermal tolerances gauged through survival experiments.
79 Moreover, a better understanding of responses to current climate change predictions for subterranean
80 animals has been identified as a fundamental question in subterranean biology given emerging
81 conservation issues associated with their respective ecosystems (29). To address this knowledge gap,
82 here we make use of genomic data from Australian representatives of a group of aquatic
83 invertebrates containing both epigean and subterranean lineages.
84 The Yilgarn Craton in central Western Australia (WA) houses a diverse subterranean diving
85 beetle fauna belonging to two tribes, Bidessini and Hydroporini (Dytiscidae). While epigean species
86 can be found practically continent-wide, subterranean taxa are isolated in calcrete aquifers (hereafter
87 calcretes) associated with ancient palaeodrainage systems in the region. These calcretes are
88 completely devoid of light and animals contained within them are assumed to have little to no access
89 to air above the water's surface (6). Each calcrete houses between one and three dytiscid species, but
90 at the time of writing only around a quarter of the ~200 known calcretes have been sampled (30–32).
91 Nonetheless, lineages in both tribes are known to have made independent yet parallel, repeated
92 transitions into underground habitats from epigean ancestors during the late Miocene to early
93 Pleistocene, likely in response to continental aridification (32–35). In each case these transitions
94 have involved the loss of eyes, pigment, and wings (30) as well as the gain of a remarkable ability to
95 respire directly from water (6). Preliminary evidence suggests that subterranean members of these

96 lineages are less tolerant of thermal extremes compared to epigean relatives (36), but the molecular
97 mechanisms underlying such tolerances–and genomic changes associated with a subterranean
98 transition in these animals more broadly–remain unknown. An investigation into the potential link
99 between the above-mentioned mode of respiration, oxygen delivery, and heat tolerance is also
100 lacking (37).

101 In the present study, we focus on two members of the Hydroporini: *Paroster*
102 *nigroadumbratus* (Clark), an epigean species endemic to South Australia, and the subterranean
103 *Paroster macrosturtensis* (Watts & Humphreys) found exclusively in a single calcrete at Sturt
104 Meadows in the Yilgarn region of WA. As subterranean dytiscids likely descended from only a
105 handful of epigean lineages, meaningful comparisons can be made between these taxa despite their
106 distributions being geographically disjunct and their divergence *ca.* 15 Mya (32). A recent study
107 showed that *P. macrosturtensis* has a reduced upper critical thermal maximum (CT_{max}) of 38.3ºC
108 compared to other epigean dytiscids (42–44.5ºC) (36), mirroring previous results for other cave
109 beetle species (23). These findings suggest *P. macrosturtensis* is unlikely to reach its thermal critical
110 maximum under current climate change predictions. However, given the thermal stability in its
111 environment, it remains unknown as to whether *P. macrosturtensis* might have a modified HSR
112 compared to its epigean relatives and if exposure to high temperatures may nonetheless induce
113 significant stress in this subterranean species. Here, we present transcriptomic data from individuals
114 of *P. macrosturtensis* and *P. nigroadumbratus* subjected to varying degrees of heat shock following
115 the results of Jones *et al*. (36). In the present study we specifically aimed 1) to generate a high-
116 quality, near-complete reference transcriptome for *P. nigroadumbratus* and 2) using this dataset,
117 characterise and compare the HSR of *P. nigroadumbratus* and *P. macrosturtensis*, specifically with
118 respect to which genes are differentially expressed and the conditions under which this occurs.
119
120

121 ## 2 | MATERIALS AND METHODS

122 ### 2.1 | Taxon sampling, experimental design, and cDNA sequencing

123 Beetle specimens (*P. macrosturtensis*, n=11; *P. nigroadumbratus*, n=18) were sourced as described
124 in a previous study and subjected to heat stress using an aquarium setup described therein (36).
125 Specimens were placed in one of three groups: in a controlled-temperature cabinet at 25ºC (hereafter
126 control), in vials within the experimental setup at 25ºC, and in vials within the experimental setup
127 ramped to 35ºC. The control temperature of 25ºC was selected by (36) to reflect the approximate
128 average groundwater temperatures of the aquifer that *P. macrosturtensis* is found in. Following
129 treatment, the individuals were placed in liquid nitrogen for RNA sequencing immediately after
130 exposure to control temperatures and thermal extremes. RNA extractions were performed using
131 single whole bodies prior to the synthesis and sequencing of barcoded cDNA samples. Quality
132 control of sequence data was performed using Trim Galore with default settings v.0.4.1
133 (bioinformatics.babraham.ac.uk/projects/trim_galore). More information regarding specimen
134 collection and husbandry, experimental design, cDNA sequencing, and phylogenetic analysis can be
135 found in the electronic supplementary material.

136

137 ### 2.2 | Assembly of *Paroster nigroadumbratus* reference transcriptome

138 A reference transcriptome for *P. nigroadumbratus* was *de novo* assembled using Trinity v.2.5.1
139 using default settings (38) and derived from unpublished raw RNA-seq data from (39). Resulting
140 transcriptome completeness was validated using BUSCO v.5 (40,41), and functional annotation was
141 performed using TransDecoder v.5.5.0 (42) and the Trinotate v.3.2.1 pipeline (38), which employs
142 SQLite (http://sqlite.org/index.html), BLAST v.2.7.1 (43), and HMMER v.3.2 (http://hmmer.org).
143 Redundancy within the transcriptome assembly was reduced by retaining only the longest isoform
144 for each Trinity gene identifier, following (10), ensuring differential expression analysis was
145 performed at the Trinity 'gene' level rather than that of isoforms. This subsetted dataset was used in

146 downstream analyses to reduce redundancy, though we note this approach limited our ability to
147 examine alternative splice variants.
148

149 ## 2.3 | Differential expression and Gene Ontology enrichment analysis

150 The above reference transcriptome was indexed and raw reads generated in this study from both *P.*
151 *macrosturtensis* and *P. nigroadumbratus* were quasi-mapped to it and normalised using Salmon
152 v.1.1.0 (44). Differential expression analysis was performed using edgeR v.3.32.1 (45) alongside
153 Gene Ontology (GO) enrichment analysis, executed using Trinity helper scripts. Differential
154 expression and enrichment/depletion of GO terms was gauged using two comparisons per species
155 (Figure 1). First, for each species the group from the experimental setup at 25ºC was compared to the
156 control group outside of the setup at 25ºC (hereafter comparison 1). Second, for each species the
157 group in the experimental setup at 35ºC was compared to the group in the experimental setup at 25ºC
158 (hereafter comparison 2). A diagrammatic representation of these comparisons is shown in Figure 1.
159 These comparisons were designed to gauge the impact of the presence of the experimental setup
160 alone and an increase in temperature, respectively. A full description of this process can be found in
161 the electronic supplementary material.
162

163 ## 2.4 | Phylogenetic analysis of putative heat shock proteins

164 Putative heat shock protein-encoding genes were aligned with coleopteran orthologs sourced from
165 the OrthoDB v.10.1 catalogue (46) and GenBank to confirm their identity (Table S1). Phylogenetic
166 inference for each HSP family was performed in RAxML v.8.2.12 (47).
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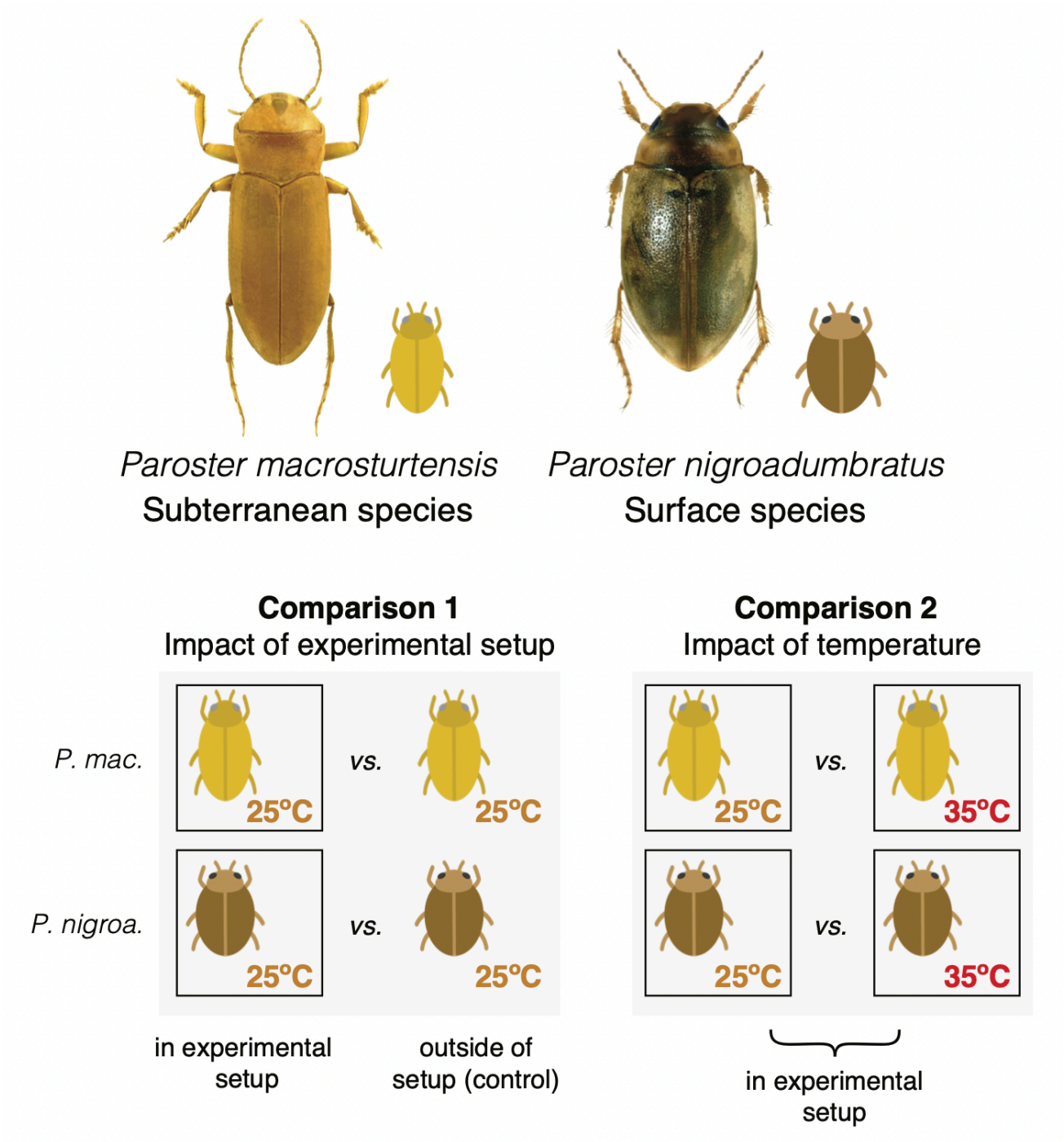

FIGURE 1 *Paroster* beetles included in this study and the experimental design used to assess differential gene expression associated with thermal extremes alone, as opposed to sole the presence of the experimental setup, following (36). Only intraspecific comparisons were made when assessing differential gene expression in our analyses; interspecific comparisons were made post-hoc. Photographs by Chris Watts and Howard Hamon.

201 # 3 | RESULTS

202 ## 3.1 | A high-quality reference transcriptome for *Paroster nigroadumbratus*
203 ## enables the characterisation of genes involved in the heat shock response

204 Here we present a high-quality, near-complete transcriptome for the epigean beetle *P.*
205 *nigroadumbratus*. This dataset consisted of 75,045,266 paired-end reads (72,266,264 following
206 quality control measures), 84% of which were incorporated into 134,246 *de novo* assembled
207 transcripts representing 60,683 unique Trinity "genes" and 41,979 predicted ORFs. According to the
208 assessment using BUSCO, this transcriptome was 87.66% complete with respect to complete core
209 arthropod genes and 96.74% complete when considering partial genes. Of these transcripts, 47,810
210 were able to be functionally annotated using the Trinotate pipeline. Subsetting our predicted peptide
211 dataset to include only the longest isoforms per Trinity gene identifier, allowing us a proxy with
212 which to perform our downstream analyses at the gene level, resulted in 14,897 predicted ORFs
213 (with 11,609, or ~77%, having some level of annotation).
214

215 ## 3.2 | Differential expression analysis reveals distinct expression profiles
216 ## associated with the heat shock response in *Paroster*

217 We compared expression profiles between members of the same species subjected to different
218 conditions to assess genes differentially expressed in response to the presence of the experimental
219 setup employed here (comparison 1) or an increase in temperature in that setup (comparison 2)
220 (Figure 2). Parallels and contrasts between *P. nigroadumbratus* and *macrosturtensis* were then
221 assessed post-hoc. Differential expression analysis using edgeR identified a total of 723 differentially
222 expressed (DE) genes in the epigean *P. nigroadumbratus* samples and 157 in the subterranean *P.*
223 *macrosturtensis*, with the two species exhibiting complex and markedly different expression profiles
224 (Figures 2, 3, S2, S3). *P. nigroadumbratus* consistently differentially expressed a greater number of

225 genes than *P. macrosturtensis*: sixty-seven and 147 genes were differentially expressed in
226 comparison 1 and 51 and 89 genes were differentially expressed in comparison 2, respectively. The
227 presence of the experimental setup and an increase in water temperature caused both species to
228 differentially express genes involved in the heat shock response. Contrasting expression profiles with
229 respect to the genes involved were not only observed between the two species (as above) but also in
230 the response of each species to these two different stressors (Figure 2).

231 In comparison 1, the surface species *P. macrosturtensis* significantly upregulated a HSP gene
232 putatively annotated as *Hsp68*, encoding a major heat shock protein in the HSP70 family, relative to
233 the control. *Paroster nigroadumbratus* upregulated the heat shock cognate *Hsc70-2* and
234 downregulated the cochaperone *l(2)efl* in the experimental setup relative to the control. None of the
235 genes differentially expressed by *P. nigroadumbratus* in response to the presence of the experimental
236 setup only were differentially expressed in by *P. macrosturtensis* or by *P. nigroadumbratus* in
237 comparison 2.

238 In comparison 2, both species upregulated the HSP genes *Hsc70-4* and *Hsp83* as well as the
239 cochaperones *Samui*, *DnaJ*, *AHSA1,* and *Unc45a* relative to groups at the lower temperature of 25ºC.
240 Both *P. nigroadumbratus* and *macrosturtensis* also upregulated separate Trinity "genes" both
241 annotated as *Hsp68* each at 35ºC relative to the 25ºC treatment (Figure 2), likely representing closely
242 related loci that are yet to be comprehensively characterised in the beetles. Additional differentially
243 expressed genes unique to each species at 35ºC relative to the 25ºC treatment included: 1)
244 downregulation of the HSP *Hsp60A* and the cochaperone *Tsc2,* and the heat shock cognate *Hsc70-2*
245 in *Paroster macrosturtensis*, and 2) the upregulation of the cochaperone *l(2)efl* and *Hsc70-3* in *P.*
246 *nigroadumbratus*. Other annotated genes potentially involved in the HSR, such as *Hsc70-2*, *Hsc70-5*,
247 *Trap1*, and *Hsp90b1*, were not differentially expressed in either species (Figure 4). A full list of
248 differentially expressed genes shared between (or unique to) the two beetle species under different
249 conditions is available in Table S2. Proteins unrelated to the HSR, yet widely differentially expressed

250 in our dataset (i.e., with reoccurring annotations across different Trinity 'genes') included those
251 involved in the transport of lipids and nutrient storage, such as vitellogenin and apolipophorins.
252 Gene Ontology enrichment analysis enables a high-order approximation of the functional
253 consequences of differentially expressed genes. We did not observe any consistent depletion of GO
254 terms associated with downregulated genes, but enriched terms were reflective of our differential
255 expression results above (Figure 3). In both species, terms associated with the HSR were enriched in
256 association with an increased temperature at 35ºC (e.g., ATPase activator activity [GO:0001671],
257 chaperone binding [GO:0051087], and unfolded protein binding [GO:0051082]). The most common
258 remaining Gene Ontology terms included lipid transporter activity (GO:0005319), nutrient reservoir
259 activity (GO:0045735), metal ion binding (GO:0046872), and zinc ion binding (GO:0008270).
260

261 **3.3 | Phylogenetic analyses of HSPs**

262 Our HSP nucleotide alignments consisted of 12 sequences across 1,743 bp (HSP60 family), 67
263 sequences across 3,669 bp (HSP70 family), 29 sequences across 3,741 bp (HSP90 family) and 23
264 sequences across 867 bp (sHSP family). Sequences clustered by gene with strong node support in our
265 phylogeny (Figure 4). All sequences from the *Paroster* species examined here were recovered as
266 nested within these clades, confirming the orthology of annotated HSPs, with the exception of a
267 transcript present in both species and inferred as sister to the *Hsp68+Hsc70-2* clades. This gene was
268 not differentially expressed in response to heat shock in our dataset.
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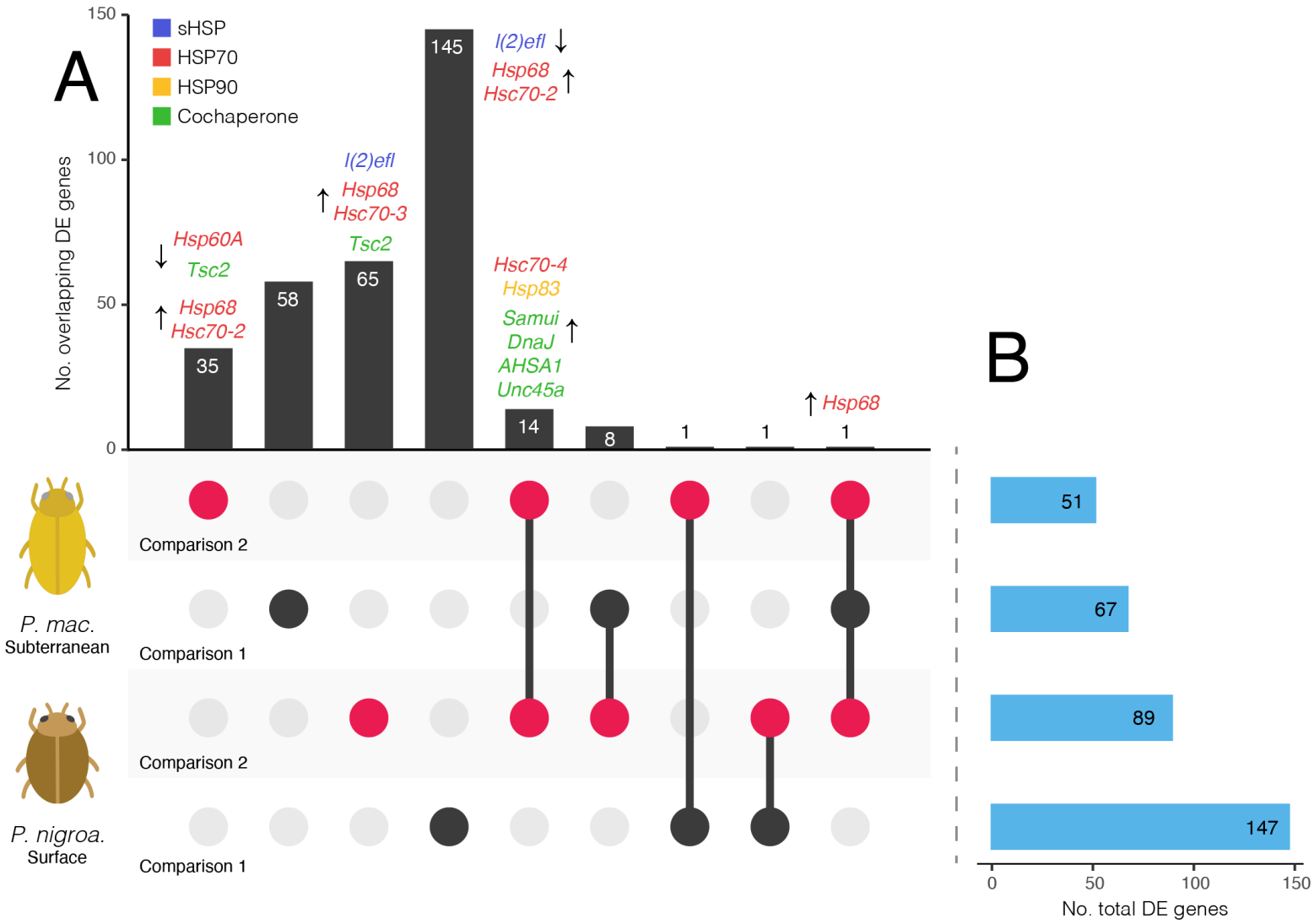

FIGURE 2 Differentially expressed (DE) genes in *Paroster macrosturtensis* and *nigroadumbratus* in comparison 1 (the experimental setup used in this study relative to the control, black circles) or comparison 2 (35ºC within the setup relative to 25ºC in the setup, red circles). Comparisons are ordered by total number of DE genes. A) DE genes shared (circles linked by lines) or unique to (unlinked circles) each comparison per species, summed in the bar graph above. Up- or downregulated HSPs or HSP cochaperones are shown for each group. The gene *Hsp68* being named more than once in different groups refers to separate transcripts sharing the same putative annotation (see Table S2). B) Total DE genes for each species under different conditions. HSP gene names were sourced from Trinotate annotations and orthology was validated using phylogenetic analysis (Figure 4).

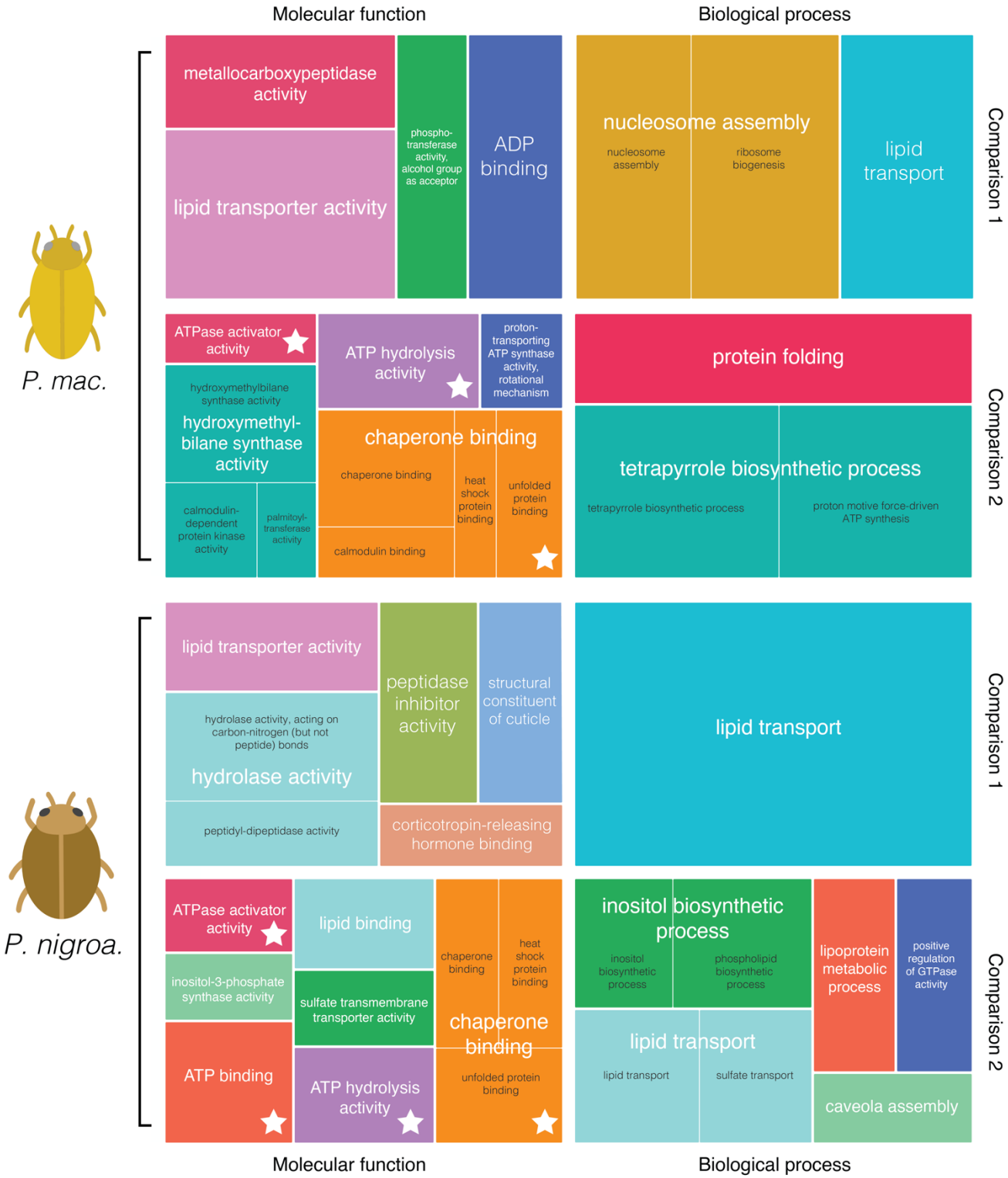

FIGURE 3 REVIGO (48) treemaps showing enriched Gene Ontology (GO) terms associated with the differential expression of genes shown in Figure 2. Treemaps are labelled as comparison 1 (the experimental setup used in this study relative to the control) or 2 (35ºC within the setup relative to 25ºC in the setup). The size of squares 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. GO terms associated with genes involved in the heat shock response are binding, indicated by a star.

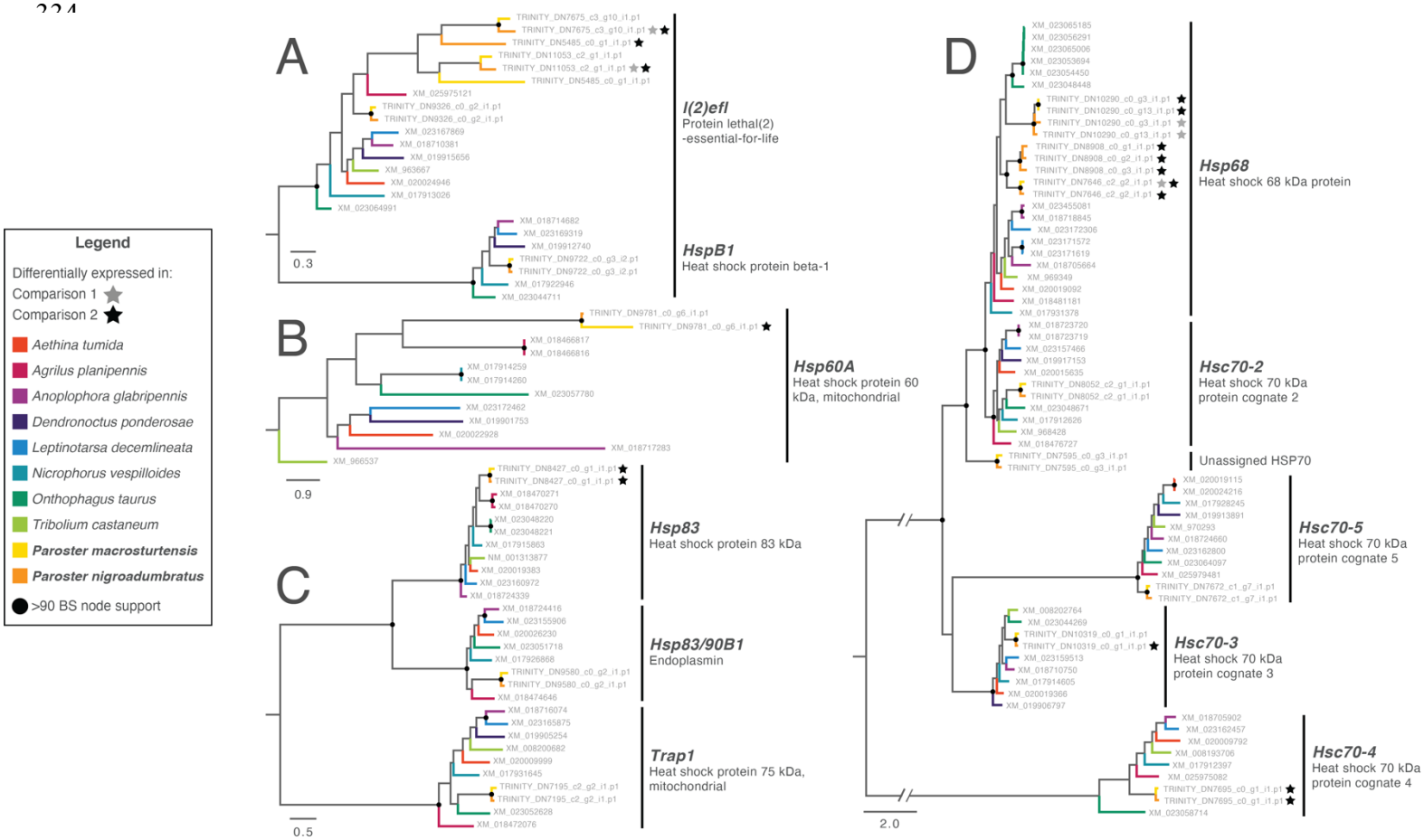

FIGURE 4 Phylogenies of heat shock Trinity 'genes' inferred using RAxML to validate the identity of putative HSP orthologs in *Paroster* species. Trees are as follows: A) sHSP family, B) HSP60, C) HSP90, D) HSP70. Scale bar is in substitutions/site; BS = bootstrap node support. Tip labels show transcript names/GenBank accession numbers. Tip names for *P. macrosturtensis* are shared with the reference *P. nigroadumbratus* transcript reads were assembled against. Tip names with stars specifically refer to genes differentially expressed the presence of the experimental setup alone relative to the control (comparison 1) or at 35ºC relative to 25ºC in the experimental setup (comparison 2).

358 # 4 | DISCUSSION

359 Here, we have comprehensively characterised the heat-shock response at the molecular level in the
360 subterranean diving beetle *Paroster macrosturtensis* and one of its surface-dwelling relatives. Using
361 a near-complete reference transcriptome for *P. nigroadumbratus*–the first such dataset for a member
362 of the Hydroporinae–we performed differential expression and GO enrichment analysis to explore
363 genes putatively involved in the HSR. Our results demonstrate that both the epigean *P.*
364 *nigroadumbratus* and subterranean *P. macrosturtensis* have an inducible HSR, in agreement with
365 implications of previous survival experiments for the genus (36). Putative orthologs of HSP
366 cochaperones, sHSP, HSP60, HSP70, and HSP90 genes were accounted for in our differential
367 expression analysis. However, the conditions under which this response is activated differs between
368 species, and *P. macrosturtensis* notably differentially expressed approximately a third of the number
369 of genes compared to *P. nigroadumbratus* in response to rising temperature.
370

371 ## 4.1 | Heat shock-induced gene expression in *Paroster*

372 HSPs identified as differentially expressed in this study support past results for beetle species and
373 other insects more broadly. A major trend in our results concerned the upregulation of HSP70 genes
374 at high temperatures, particularly *Hsp68*; HSP70s are highly expressed in response to heat shock in
375 other beetle species, including cave-adapted subterranean taxa (28,49,50), and work in concert with
376 sHSPs and HSP90s (51–54). In addition to the heat shock proteins, we also observed the
377 upregulation of the heat shock cognates *Hsc70-2*, *Hsc70-3,* and *Hsc70-4* and cochaperones *Tsc2,*
378 *Samui*, *DnaJ*, *AHSA1*, *Unc45a* in at 35ºC relative to the 25ºC treatment. To our knowledge there has
379 been no documentation of a coleopteran heat shock cognate being upregulated in response to
380 increasing temperature, though evidence exists for the parasitic wasp *Pteromalus*, in which *hsc70* is
381 also induced by heavy metal poisoning and starvation in addition to thermal extremes (55). Heat
382 shock cognates are also upregulated during diapause in silkworm eggs (56) and young bumble bee

383 queens (57), potentially playing a cryoprotective role in these species. Cochaperones are less well
384 characterised in insects, but evidence for their upregulation in response to heat shock has been
385 documented in hemipterans and ants (58–60). We observed the downregulation of several heat shock
386 proteins in both species in the presence of the experimental setup relative to the control, and at high
387 temperatures relative to the 25ºC treatment. Both sHSPs and HSP70s have been documented as being
388 downregulated during periods of heat stress in other insects, e.g. in silk moths (61). In *P.*
389 *nigroadumbratus* this was restricted to the sHSP *l(2)efl* in the experimental setup-only comparison.
390 In contrast, the cochaperone *Tsc2* was downregulated *in P. macrosturtensis* at high temperatures
391 relative the 25ºC treatment.
392

393 **4.2 | Expression profiles reflect differing thermal tolerances**

394 Our molecular data mirrors previously documented reduced thermal tolerances in subterranean
395 insects such as *P. macrosturtensis*. The species differentially expressed far fewer genes in response
396 to 35ºC relative to the 25ºC treatment compared to *P. nigroadumbratus*; similarly reduced numbers
397 of differentially expressed genes have also been associated with lower thermal tolerances in other
398 organisms such as fish (62), lizards (63), rotifers (64), red algae (65), and plants (66,67), though we
399 note the inverse (or alternatively, no clear pattern) has been observed in a number of cases,
400 potentially reflecting lower levels of stress as opposed to an inability to mount a HSR (68,69).
401 In keeping with the above findings, *P. macrosturtensis* also differentially expressed far fewer
402 genes than its epigean counterpart in response to the presence of the experimental setup alone
403 relative to the control (Figure 2). HSPs are known to be involved in responding to a wide range of
404 stressors (70–72), and the involvement of such genes is not surprising in stress unrelated to
405 temperature; individuals being moved into the experimental setup employed here may have induced
406 stress from handling, for example. The greater number of genes differentially expressed by *P.*
407 *nigroadumbratus* in this scenario may suggest *P. macrosturtensis* is potentially less able to robustly

408 respond to ambient stressors more broadly (i.e., environmental disturbances). Such a scenario is
409 supported by past work showing subterranean species are sensitive to ambient stressors under
410 otherwise non-stressful temperatures (73), and being an epigean species, *P. nigroadumbratus* is
411 presumably exposed to far more dramatic environmental fluctuations (in addition to more variable
412 temperatures) on a regular basis than a subterranean species such as *P. macrosturtensis*. We also note
413 that in the presence of the experimental setup relative to the control, *P. macrosturtensis* upregulated
414 the same *Hsp68*-annotated Trinity 'gene' implicated in responses to heat-induced stress in both
415 species (Figure 2), whereas HSPs differentially expressed by *P. nigroadumbratus* did not overlap
416 with those in other groups.
417

418 **4.3 | Heat shock and the climatic variability hypothesis**

419 The dataset we present here adds to a growing body of knowledge concerning the HSR in organisms
420 that inhabit thermally stable environments. Central to discourse on this topic is the climatic
421 variability hypothesis, which posits that the thermal tolerance of a taxon is positively correlated with
422 its temperature ranges encountered in nature (74). This hypothesis implies species from extremely
423 stable thermal environments can no longer tolerate temperature extremes, and has been demonstrated
424 in a wide variety of organisms that have either lost or possess a reduced HSR, such as cnidarians
425 (75), limpets (76), amphipods and sea stars (77), and midges (78). In contrast, species that inhabit
426 areas with a broader range of climatic conditions would be expected to be more robust in the face of
427 environmental fluctuations (79). While *P. macrosturtensis* does have a lower thermal tolerance
428 compared to *P. nigroadumbratus*, in line with the above hypothesis, it nonetheless has retained a
429 HSR at high temperatures per our transcriptomic data. Similar studies have shown certain
430 groundwater-dwelling organisms display an inducible HSR in response to conditions far warmer than
431 they would encounter in nature (20,21). The HSR of these species, as well as *P. macrosturtensis*,
432 might be retained at such high temperatures for a variety of reasons, including the fact that the

433 species has not occupied its respective environments for a sufficient length of time in evolutionary
434 terms for their HSR to be lost, e.g. via adaptive processes or a relaxation of purifying selection (36).
435 The latter scenario is plausible as *P. macrosturtensis* is also known to have retained the ability to
436 detect light despite inhabiting an aphotic environment for over ~3 million years (80).
437

438 **4.4 | Conservation implications**

439 While the retention of a HSR in both species examined here supports the physiological findings of
440 Jones *et al*. (36), almost half (4 out of 10 assayed) of the *P. macrosturtensis* cohort did not survive 24
441 hours after heat shock in that study. It therefore remains to be seen if the species can tolerate such
442 extremes in the long term. Indeed, even cave beetles considered stenothermal–those that are only
443 capable of surviving within an extremely narrow temperature range–have retained the HSR (23), but
444 nonetheless cannot survive at extreme temperatures for long periods (>7 days) compared to epigean
445 relatives (25,28). Threatening processes that *P. nigroadumbratus* and *macrosturtensis* are both at risk
446 of experiencing in their fragile habitats might impact the latter species far more negatively as a
447 result.

448 Temperature rises of up to 5ºC by the end of the century compared to pre-industrial levels
449 may occur in central Western Australia per current climate change projections (81). Water
450 temperatures in aquifers are generally cooler and more stable than, but are nonetheless coupled with,
451 conditions above-ground, and are also predicted to warm as regional temperatures increase (82–84).
452 The subterranean habitat of these insects is therefore unlikely to shield them from the impacts of a
453 warming world. The fact that *P. macrosturtensis* appears to be unable to mount as robust a HSR
454 compared to *P. nigroadumbratus*, and therefore may experience a significantly higher amount stress
455 compared to epigean species in the face of high temperatures, has conservation implications for the
456 understudied fauna of the Australian Yilgarn and beyond.

457 Datasets such as these are especially pertinent for subterranean invertebrates found in the
458 Yilgarn region—including *P. macrosturtensis* and its subterranean relatives, in addition to
459 crustaceans such as isopods and amphipods—as the groundwater in their calcrete habitats is heavily
460 utilised for water extraction by industry (85,86). As short-range endemics to the extreme, such
461 species are not only at risk of habitat degradation via climate change, but from the direct intersection
462 of shallow aquifers with e.g. mining activities and through the drawdown of groundwater beneath
463 calcretes at greater depths (87). In addition to reflecting the reduced thermal tolerances of Australian
464 subterranean dytiscids, the molecular data we presented here for *P. macrosturtensis* also suggests a
465 potentially weaker response in the face of other environmental disturbances unrelated to temperature.
466 These factors have the potential to render *P. macrosturtensis* more vulnerable to both of the above
467 threatening processes compared to epigean relatives, with implications for subterranean fauna more
468 broadly. An increased knowledge of the assumed fragility of Australian subterranean invertebrates in
469 the face of these stressors is therefore crucial for informing future conservation management plans
470 for these animals and their fragile habitats.
471

472 **5 | CONCLUSIONS**

473 Our findings demonstrate the reduced thermal tolerance of the subterranean species *P.*
474 *macrosturtensis* compared to its epigean relatives is reflected, and further clarified by, transcriptomic
475 data. While our data are supported by past physiological evidence that demonstrated *P.*
476 *macrosturtensis* could survive at high temperatures, albeit not to the limits of epigean species (36),
477 the present study adds a new layer to this narrative. *P. macrosturtensis* might possess increased
478 mortality in the face of high temperatures because the species differentially expresses far fewer genes
479 in response to heat shock compared to the epigean relative *P. nigroadumbratus*, suggesting it may be
480 unable to mount as robust a heat shock response. While *P. macrosturtensis* might be able to survive
481 at temperatures far above those it encounters in nature for short periods, as detailed by Jones *et al*.

482 (36), our results suggest the species also experiences a weaker transcriptomic response to factors
483 unrelated to temperature (i.e. the presence of the experimental setup employed here) relative to *P.*
484 *nigroadumbratus*. As such, *P. macrosturtensis* may not be as well-equipped to survive higher
485 temperatures and other threatening processes, such as disturbances to surrounding groundwater, in
486 the long term compared to surface-dwelling members of *Paroster*. Future work in this system will
487 ideally assess a far greater number of dytiscid species to further explore the trends we observe here.
488 As the present study did not consider the role of isoforms in the heat shock response of these
489 animals, broader studies could examine these responses to heat stress at a finer scale by conducting
490 differential expression analysis on the transcript, as opposed to gene, level.
491

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AUTHOR CONTRIBUTIONS AND COMPETING INTERESTS STATEMENT

PGBH, KJ, and SJBC conceived the study. KJ performed experiments exposing beetles to heat stress. PGBH analysed the data and wrote the manuscript. TB and CSPF provided guidance regarding bioinformatic analyses. ST performed molecular laboratory work. TMB, KJ, WFH, and ADA helped to draft the manuscript. All authors edited the manuscript and approved the final draft. Funding for this project was provided by an Australian Research Council Discovery grant (DP180103851) to SJBC, WFH, ADA and TB. The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

All raw RNA-seq data for *Paroster macrosturtensis* and *P. nigroadumbratus* used in differential expression analyses in this study are available via NCBI under BioProject PRJNA783065 (individual Sequence Read Archive accessions for samples SRR17023302-SRR17023315). HSP transcripts assembled from raw *P. macrosturtensis* RNA-seq data and used in phylogenetic analysis are available via GenBank (accession numbers summarized in Table S1). The reference transcriptome of *P. nigroadumbratus* (including all isoforms) and the annotated, subsetted dataset (only including longest isoform per Trinity ‘gene’) are available via FigShare (DOI: 10.25909/17169191).