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 epigeal) habitats. While epigeal 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).

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 (Somero, 2020; Tomanek, 2010).

Knowledge of the HSR in organisms from thermally stable *subterranean* habitats, including their associated inducible HSPs and at which temperatures this response might be activated, is almost non-existent (e.g. Colson-Proch *et al.*, 2010). The vast majority of the literature on this topic is also devoted to vertebrates, in stark contrast to their relatively small contribution to the biodiversity of subterranean habitats, which is dominated by invertebrate taxa (Gibert & Deharveng, 2002; Humphreys, 2012). Moreover, a better understanding of responses to current climate change predictions for subterranean animals has been identified as a fundamental question in subterranean biology given emerging conservation issues associated with their respective ecosystems (Mammola *et al.*, 2020). To address this knowledge gap, here we make use of genomic data from Australian representatives of a group of aquatic invertebrates containing both epigeal and subterranean lineages.

The Yilgarn Craton in central Western Australia houses a diverse subterranean diving beetle fauna belonging to two tribes, Bidessini and Hydroporini (Dytiscidae). While epigeal species can be found practically continent-wide, subterranean taxa are isolated in calcrete aquifers (hereafter calcretes) associated with ancient palaeodrainage systems in the region. These calcretes are completely devoid of light and animals contained within them are assumed to have little to no access to air above the water's surface (Jones *et al.*, 2019). Each calcrete houses between one and three dytiscid species, but at the time of writing only around a quarter of the ~200 known calcretes have been sampled (Cooper *et al.*, 2002; Leijts *et al.*, 2012; Watts & Humphreys, 2009). Nonetheless, lineages in both tribes are known to have made independent yet parallel, repeated transitions into underground habitats from epigeal ancestors during the late Miocene to early Pleistocene, likely in response to continental

aridification (Langille et al., 2021; Leijs et al., 2012; Leys et al., 2003; Toussaint et al., 2015). In each case these transitions have involved the loss of eyes, pigment, and wings (Cooper *et al.* 2002) as well as the gain of a remarkable ability to respire directly from water (Jones *et al.* 2019). Preliminary evidence suggests that subterranean members of these lineages are less tolerant of thermal extremes compared to epigeal relatives (Jones et al., 2021), but the molecular mechanisms underlying such tolerances—and genomic changes associated with a subterranean transition in these animals more broadly—remain unknown.

In the present study, we focus on two members of the Hydroporini: *Paroster nigroadumbratus* (Clark), an epigeal species endemic to South Australia, and the subterranean *Paroster macrosturtensis* (Watts & Humphreys) found exclusively in a single calcrete (hereafter the Sturt Meadows calcrete) in the Yilgarn region of Western Australia. As subterranean dytiscids likely descended from only a handful of epigeal lineages (Leijs et al., 2012), meaningful comparisons can be made between these taxa despite their distributions being geographically disjunct. A recent study showed that *P. macrosturtensis* has a reduced upper thermal tolerance (or critical thermal maximum, CT_{max}) of 38.3°C compared to other epigeal dytiscids (42–44.5°C) (Jones et al., 2021), mirroring previous results for other cave beetle species (Pallarés et al., 2020). These results also suggest *P. macrosturtensis* is unlikely to reach its thermal critical maximum under current climate change predictions. However, given the thermal stability in its environment it remains unknown as to whether *P. macrosturtensis* might have a modified HSR compared to its epigeal relatives. Building on this previous work, here we present transcriptomic data from individuals of *P. macrosturtensis* and *P. nigroadumbratus* previously subjected to varying degrees of heat shock by Jones *et al.* (2021). In the present study we specifically aim 1) to generate a high-quality, near-complete reference transcriptome for *P. nigroadumbratus* and 2) using this dataset, characterise and compare the HSR of *P. nigroadumbratus* and *P. macrosturtensis*,

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

2 | METHODS

2.1 | Taxon sampling and experimental design

Beetle specimens were sourced from a previous study by Jones *et al.* (2021) that subjected specimens to heat stress. Briefly, *P. macrosturtensis* samples (n = 11) were collected from the Sturt Meadows calcrete in WA via existing mineral exploration boreholes. Epigeal *P. nigroadumbratus* specimens were collected from water bodies in the Mount Crawford region of South Australia (n = 18). Individuals were kept by Jones *et al.* (2021) in constant temperature cabinets at between 24 and 26°C either in a 12:12h light-dark cycle (epigeal species) or in complete darkness (subterranean species) for at least seven days prior to subsequent experimentation to minimise the effects of variable thermal history and stress during capture and transport. Beetles were then either retained in this constant temperature environment and euthanized in liquid N₂ (control treatment) or moved to a temperature-controlled water bath containing pure reverse osmosis (RO) water (for epigeal species) or RO water with a salinity level mimicking calcrete microhabitats (for subterranean species). Beetles were kept at 25°C in this environment for 4.5 hours to determine if they could survive the experimental setup (25°C treatment) or allowed to acclimate to 25°C for 1 hour before the temperature was increased by 0.05°C every minute until it reached 35°C (35°C treatment). In all cases individuals were euthanized in liquid N₂ immediately after this time period.

2.2 | cDNA synthesis and sequencing

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

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

2.3 | Quality control of sequence data

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

2.4 | Assembly of *Paroster nigroadumbratus* reference transcriptome

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

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

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

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

2.5 | Differential expression analysis and Gene Ontology enrichment analysis

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

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

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

0.9. Resulting tree maps were produced with values set to $\text{abs}(\log_{10}(p\text{-value}))$ to ensure plots reflected the significance of enrichment of each term.

2.6 | Phylogenetic analysis of putative heat shock proteins

Genes annotated as putatively encoding heat shock proteins consisted of members of three of the four insect families of HSP, including small heat shock proteins (sHSP), HSP70, and HSP90. Transcripts were extracted on the basis of annotations containing the terms *heat shock*, *HSC*, *HSP*, or *chaperone*. To ensure the inclusion of as many genes as possible for which annotation might have been incomplete (e.g. certain HSP chaperones), we also included transcripts putatively involved in the HSR that were differentially expressed in our dataset yet not captured in the above search. The resulting candidate set of 38 transcripts was checked against the UniProt database to confirm the function of the proteins they encoded, and the identities of transcripts were then validated using BLASTN searches restricted to the Coleoptera against the NCBI nr database. These transcripts were used as a reference to filter raw reads from each *P. macrosturtensis* 35°C replicate using BWA v.0.7.17 (H. Li & Durbin, 2009) and SAMtools v.1.9 (H. Li et al., 2009). Filtered *P. macrosturtensis* reads (excluding singletons) were then pooled and mapped against reference transcripts using the Read Mapper tool in Geneious Prime (v.2021.2.2, [geneious.com](https://www.geneious.com)) with Low Sensitivity presets. Consensus sequences for *P. macrosturtensis* were produced from these alignments with a 0% (majority) threshold.

Coleopteran orthologs of the above genes were retrieved from OrthoDB v.10.1 (Kriventseva et al., 2019) with LOC identifiers of the top BLASTN hit as queries. mRNA transcripts of protein sequences in question were obtained from GenBank, representing sequences from *Aethina tumida* Murray (Polyphaga: Cucujoidea), *Agilus planipennis* Fairmaire (Polyphaga: Buprestoidea), *Anoplophora glabripennis* (Motschulsky) (Polyphaga:

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

3 | RESULTS

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

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

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

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

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

the HSR, such as *Hsc70-2*, *Hsc70-5*, *TRAP1*, and *Hsp90b1*, were not differentially expressed in either species. A full list of differentially expressed genes shared between (or unique to) treatments is available in Table S2.

Gene Ontology enrichment analysis enables the visualisation and collapse of similar GO terms associated with differentially expressed genes. We did not observe any consistent depletion of GO terms associated with downregulated genes, but enriched terms were reflective of our differential expression results above (Figure 2). In both species, terms associated with the HSR were enriched at 35°C (e.g. ATPase, chaperone binding, and unfolded protein binding). At 25°C, ADP binding was also enriched for *P. macrosturtensis* due to the upregulation of *Hsp68* at that treatment.

3.3 | Phylogenetic analyses

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

4 | DISCUSSION

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

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

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

HSPs identified as differentially expressed in this study support past results from beetle species and other insects more broadly. A major trend from our results concerned the upregulation of HSP70 genes, particularly *Hsp68*; HSP70s are highly expressed in response to heat shock in the Colorado potato beetle (Yocum, 2001), red flour beetle (Chen et al., 2020), and the cave beetle *Neobathyscia* (Bernabò et al., 2011). These genes are upregulated in concert with sHSPs and HSP90 genes in the rice weevil (Yuan et al., 2014) and Japanese sawyer beetle (Cai et al., 2017) under the same conditions. HSP70s and sHSPs are also known to be upregulated in response to heat shock in the cigarette beetle (M.-Y. Li et al., 2021; W.-J. Yang et al., 2019).

In addition to the heat shock proteins, we also observed the upregulation of the heat shock cognate *Hsc70-4* and cochaperones *DnaJ*, *Samui*, *BAG2*, and *AHSA1* in response to heat shock in *Paroster*. To our knowledge there has been no documentation of a coleopteran heat shock cognate being upregulated in response to increasing temperature, though evidence exists for the parasitic wasp *Pteromalus*, in which *hsc70* is also induced by heavy metal poisoning and starvation in addition to thermal extremes (H. Wang et al., 2012). Heat shock

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

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

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

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

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

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

necessarily imply *Hsp68* was upregulated in response to heat stress at 25°C, as HSPs are known to be involved in responding to a wide range of stressors (King & MacRae, 2015; Sang et al., 2012; Sørensen et al., 2003) 25°C is within the range of temperatures *P. macrosturtensis* might encounter in nature (average 23°C–26.2°C) (Jones et al., 2021), and this was the same temperature ($\pm 1^\circ\text{C}$) as the control treatment of Jones *et al.* (2021). On the other hand, the upregulated *Hsp68* transcript variant was also differentially expressed in the two 35°C treatments in both species, potentially suggesting a link to heat stress.

The above could indicate *P. macrosturtensis* 1) did experience some initial degree of heat shock response in the control treatment and this was further exacerbated by the addition of the experimental setup in the 25°C treatment, or 2) the response was triggered by stress induced by being moved to designated 25°C vials, given *P. nigroadumbratus* also exhibits differential expression of HSPs at 25°C (though the downregulation of transcripts therein are not shared with other treatments) (Figure 1). The former scenario would reflect the reduced thermal tolerance of *P. macrosturtensis*, which is also supported by the species differentially expressing a far lower number of genes in response to higher temperatures compared to *P. nigroadumbratus*. Similarly reduced numbers of differentially expressed genes have also been associated with lower thermal tolerances in other organisms such as fish (Narum & Campbell, 2015), lizards (Akashi et al., 2016), rotifers (Paraskevopoulou et al., 2020), red algae (W. Wang et al., 2018), and plants (Jin et al., 2020; Sun et al., 2021), though we note the inverse (or alternatively, no clear pattern) has been observed in a number of cases, potentially reflecting lower levels of stress as opposed to an inability to mount a HSR (DeBiasse & Kelly, 2016; Perez et al., 2021).

Acquiring data such as these is not only necessary to better understand the HSR in subterranean animals in the face of climate change, but also to inform future conservation decisions. The latter is of particular relevance to members of subterranean *Paroster* as the

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

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

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

5 | CONCLUSIONS

The present study contrasted differentially expressed genes involved in the heat shock response (HSR) of *Paroster nigroadumbratus* and *P. macrosturtensis*, epigeal and subterranean lineages of hydroporine diving beetles endemic to Australia previously subjected to heat shock by Jones *et al.* (2021). Our results have comprehensively characterised the genes involved in the HSR in these species and demonstrate that the subterranean *P. macrosturtensis* retains a HSR at the molecular level, in agreement with the physiological data of Jones *et al.* (2021) detailing that the species can survive temperatures far above what it would encounter in nature. However, we also show that *P. macrosturtensis* is potentially less equipped at the molecular level to respond to higher temperatures, with the species differentially expressing less than a fifth of the number of genes in response to heat shock in *P. nigroadumbratus* at 35°C. Together, these results support the idea that *P. macrosturtensis* has lost some degree of tolerance to temperature extremes compared to epigeal species such as *P. nigroadumbratus*, in agreement with the climatic variability 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).

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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 25°C and 35°C treatments vs. control treatment (25°C 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 25°C and 35°C 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.