Molecular adaptations to heat stress in the thermophilic ant genus *Cataglyphis*

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Running title: Molecular adaptations to heat in *Cataglyphis*

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Abstract

Over the last decade, increasing attention has been paid to the molecular adaptations used by organisms to cope with thermal stress. However, to date, few studies have focused on thermophilic species living in hot, arid climates. In this study, we explored molecular adaptations to heat stress in the thermophilic ant genus *Cataglyphis*, one of the world's most thermotolerant animal taxa. We compared heat tolerance and gene expression patterns across six phylogenetically distant species that live in different habitats and experience different thermal regimes. We found that all six species had similar heat tolerance levels and critical thermal maxima. Furthermore, the transcriptome analyses revealed that, although the number of differentially expressed genes varied widely for the 6 species (from 54 to 1,118), many were also shared. Functional annotation of the differentially expressed and co-expressed genes then showed that the biological pathways involved in heat-shock responses were similar among species and were associated with four major processes: the regulation of transcriptional machinery and DNA metabolism; the preservation of proteome stability; the elimination of toxic residues; and the maintenance of cellular integrity. Overall, our results suggest that molecular responses to heat stress have been evolutionarily conserved in the ant genus *Cataglyphis* and that their versatility may help workers withstand temperatures close to their physiological limits.

Keywords: Thermotolerance, CT_{max}, heat stress, molecular adaptation, RNA-seq, ants

Introduction

Thermal stress is a daunting physiological challenge for living organisms (Evgen'ev, Garbuz & Zatsepina, 2014). Extreme temperature variation disturbs the thermodynamic equilibria of chemical bonds and alters the three-dimensional structure of macromolecules, leading to their denaturation (Quinn, 1988; Feder & Hofmann, 1999; Wang, Lim & Son, 2014). This instability in macromolecule dynamics hinders protein functioning, results in dysregulated cell membrane fluidity, and increases the production of toxic reactive oxygen species (ROS), ultimately leading to cell malfunction and death (Evgen'ev et al., 2014; Hazel, 1995; Birben et al., 2012). To limit the negative effects of thermal stress, the cellular machinery builds on increased production of proteins that can limit and repair cell damage. Among the latter are heat-shock proteins (HSPs), which are traditionally associated with the cellular heat-shock response (HSR) (Richter, Haslbeck & Buchner, 2010). In addition to their housekeeping role, HSPs perform several essential functions during heat stress: they ensure the correct folding of other proteins, prevent dysfunctional protein aggregation (i.e., due to the association of misfolded proteins), and help eliminate protein aggregates (Evgen'ev et al., 2014). In addition to HSPs, there are other proteins and molecular pathways that are differentially regulated in response to thermal stress. For instance, higher temperatures trigger the production of long-chain unsaturated fatty acids and the incorporation of sterols into cell membranes, constraining increases in fluidity (Hazel, 1995). Likewise, the accumulation of ROSs can be dampened via the production of antioxidants and detoxification enzymes, such as super oxide dismutase (SOD) or glutathione peroxidase (GPx) (Birben et al., 2012). Over the last decade, an increasing number of studies have investigated HSRs in both vertebrates and invertebrates (reviewed in Logan & Buckley, 2015; Heikkila, 2017; Lockwood, Connor & Gracey, 2015; Perez & Aron, 2020). However, most of this research has been devoted to mesophilic species living in environments with moderate temperatures; in contrast, few studies have looked at thermophilic species living in hot, arid climates.

In this study, we examined molecular adaptations to heat stress in a group of highly thermophilic animals: *Cataglyphis* desert ants. The ant genus *Cataglyphis* is found in arid regions within the Palearctic zone (from the Mediterranean to the driest desert of Mongolia), and workers in species of this taxon can face incredibly hot and dry thermal conditions. For example, in the Sahara Desert, workers of the Saharan silver ant, *C. bombycina*, leave the nest during the hottest part of the day, when ground and air temperatures exceed 50°C and 45°C, respectively (Wehner, March & Wehner, 1992; Gehring & Whener, 1995; Cerdá, Retana & Cros, 1998). They have a tremendous competitive advantage because they can forage under climatic conditions that none of their potential competitors or predators can withstand (Wehner et al., 1992). Species occurring in more mesic habitats, like *C. piliscapa* (previously *C. cursor*), have workers who display a wider range of thermal activity than do sympatric ant species (Cerdá et al., 1998). To endure the harsh conditions of their environment, *Cataglyphis* ants have evolved a range of remarkable behavioural, morphological, and physiological adaptations (reviewed in Boulay et al., 2017). Workers actively exploit thermal refuges—such as shadows or elevated points within the landscape—to convectively cool. Their long legs maintain their bodies up off the burning ground and allow workers to run very rapidly, also enhancing convective cooling (Figure 1) (Sommer & Wehner, 2012; Pfeffer et al., 2019). *Cataglyphis bombycina* sports densely packed, prism-shaped hairs on its body that reflect light within the visible and near-infrared spectra, providing natural solar heat shielding (Shi et al., 2015; Willot et al., 2016). Surprisingly, despite their ability to withstand body temperatures close to their physiological limits (Boulay et al., 2017), the molecular responses of *Cataglyphis* ants to heat stress remain poorly studied. The role played by HSPs in such heat tolerance mechanisms has been documented in only two species: *C. bombycina*, which occurs in the Sahara Desert, and *C. mauritanica*, which occurs in semi-arid mountainous regions (Gehring & Whener, 1995; Willot, Gueydan & Aron, 2017; Willot et al., 2018). In both species, heat stress causes the upregulation of genes in the *hsp70* family. In *C. bombycina,* these genes are specifically involved in the protection of mitochondria (*hsc70-5*), the organisation of sarcomeres (*unc-89)*, and the safeguarding of cytoskeletal fibres via the action of small HSPs

105 (sHSPs), which reveals the importance of maintaining proper motor function, as the ants
106 forage at high speeds on the burning ground.

107 We compared heat tolerance and gene expression patterns across six phylogenetically
108 distant *Cataglyphis* species that live in different habitats and experience different thermal
109 regimes (Figure 2). First, we explored heat tolerance by measuring worker survival under
110 conditions of heat stress. We also investigated the ability of workers to acquire
111 thermotolerance via heat hardening. Second, we examined the differential expression and co-
112 expression of genes for control versus heat-stressed workers across species.

Materials and methods

Ant sampling and rearing conditions

We studied six species found in different climatic zones (Figure 2). Two species occur in arid, sandy deserts: *C. bombycina* (Morocco: 30°33′22″ N, -5°83′83″ E) and *C. holgerseni* (Israel: 30°41'22'' N, 35°14'14'' E); two species occur in semi-arid, mountainous regions: *C. mauritanica* (Morocco: 33°25'29'' N, -5°8'25'' E) and *C. cubica* (Morocco: 32°'4'' N, 6°12'29'' E); and two species are found along the Mediterranean coast: *C. piliscapa* (France: 42°47'34'' N, 2°59'3'' E) and *C. niger* (Israel: 32°7'18'' N, 34°47'4'' E). In these climatic zones, mean annual temperature (AMT) ranges from 10.4 to 22.9°C, and the highest temperature during the warmest month of the year (MaxT) ranges from 29.0 to 42.3°C (Figure 2).

We excavated 4–6 colonies of each species and brought them back to the lab. The colonies were reared under controlled conditions (mean temperature: 25°C [± 1°C], light-dark cycle: 12 h/12 h, and relative humidity: 30–40%). Ants were given sugar solution *ad libitum* and were fed sliced mealworms three times per week. Colonies experienced these conditions for at least one month before the experiments began.

Assessing heat tolerance and heat-hardening capacity

Ant heat tolerance was characterised using a heat-stress experiment. It has been shown that body size influences heat tolerance in ants and other insects (reviewed in Perez & Aron, 2020; Buxton et al., 2021). To a large extent, this relationship is due to differences in relative water loss (Hood & Tschinkel, 1990; Cerda & Retana, 2000; Clémencet et al., 2010). For each species, 6 groups of 10 randomly selected workers were formed; these groups were then placed in glass tubes containing a wet cotton ball to prevent ant desiccation. The glass tubes were immersed in a digitally controlled water bath kept at a constant temperature (SW22, Julabo GmbH, Seelbach, Germany). In the heat-stress (HS) treatment, trials were conducted using five different temperatures: 41°C, 43°C, 45°C, 47°C, and 49°C. In the no-heat-stress (NHS) treatment, the same general procedure was used, but there was only one temperature:

25°C. The temperature within the tubes was monitored using 0.075-mm-diameter thermocouples (Type K Thermocouple [Chromel/Alumel], RS Components Ltd, UK) connected to a digital thermometer (RS Pro RS52 Digital Thermometer, RS Components Ltd, UK). Percent survival after 3 hours was recorded; workers were classified as dead once they lost their locomotor ability (i.e., muscular paralysis). Previous studies have shown that 3 hours of stress is enough to induce a significant heat-shock response in the honeybee *Apis mellifera* (Ma et al., 2019; Zhang et al., 2019) and in the ant *C. bombycina* (Willot et al., 2018). For each species, we defined the critical thermal maximum (CT_{max}) as the temperature below which percent survival dropped considerably. Following the treatment, living workers were immediately frozen in liquid nitrogen and then stored at -80°C until the transcriptome analyses could be performed.

To test whether heat hardening increased heat tolerance, we used the procedure described in Willot et al. (2017). First, for each species, we used the results from the heat-stress experiment to determine the temperature at which percent survival was approximately 50% (T_{50}). In the heat-hardening experiments, workers were exposed to a temperature that was 8°C below T_{50} for 2 hours. Although physiologically stressful, these thermal conditions did not lead to a loss in muscular coordination. Then, the workers were subjected to more intense heat stress: they were placed in another water bath kept at T_{50} for 3 hours. The mean percent survival of heat-hardened workers (2 hours at T_{50} minus 8°C + 3 hours at T_{50}) and non-heat-hardened workers (3 hours at T_{50}) was compared using *t*-tests, after confirming the normality and homoscedasticity of the residuals. We also performed Kaplan-Meier survival analysis. All statistical analyses were carried out using R (v. 4.0; R Development Core Team 2017).

RNA-seq library preparation and sequencing

RNA extraction, RNA quality (i.e., determined via Bioanalyzer), and RNA-seq library preparation and sequencing were performed by BGI Tech Solutions (Hong Kong). Total RNA was extracted from whole ant bodies using Trizol (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. To analyse patterns of differential gene

expression, four replicates were used to represent each heat-stress treatment (HS = workers at CT_{max} for 3 hours vs. NHS = workers at 25°C for 3 hours). Sequencing was performed using an Illumina HiSeq 4000 System. Each replicate contained the entire bodies of 10 workers from the same colony. About 25 million single reads of 50 bp in length were generated per sample. Since no reference transcriptome was available for the *Cataglyphis* taxa used in this study, we also sequenced a pool of workers (HS = 5 and NHS = 5), eggs, and pupae for each species using a HiSeq X Ten System; the resulting transcripts were used in *de novo* transcriptome assembly. About 90 million paired reads of 150 bp in length were obtained for each species pool.

Transcriptome assembly and analyses of differential gene expression

The transcriptome analyses were species specific and were carried out following the same procedure. The quality of all the sequenced reads was estimated using FastQC (v. 0.11.7; Andrews, 2010). To assemble the reference transcriptome, we used the set of sequences obtained from the pool of workers, eggs, and pupae. First, we digitally normalised the reads (20x coverage). Second, we assembled the reads using the Trinity pipeline (v. 2.8.4; Grabherr et al., 2011) and two independent strategies: full *de novo* assembly and reference-guided assembly. The reference-guided approach utilised the genome of *Cataglyphis hispanica* (unpublished data). These two independently assembled transcriptomes were then merged, and redundant transcripts were removed using CD-Hit (v. 4.8.1; Huang et al., 2010), applying a threshold of 95% nucleotide similarity. Finally, transcripts were clustered into superTranscripts via Corset (v. 1.08; Davidson & Oshlack, 2014) and Lace (v. 1.13; Davidson, Hawkins & Oshlack, 2017), which further reduced transcript redundancy by improving posterior gene expression counts (Davidson et al., 2017). This assembly was then annotated using Annocript (v. 2.0.1; Musacchia et al., 2015) in tandem with the UniProt Reference Cluster (UniRef90) and UniProtKB/Swiss-Prot (Suzek et al., 2015) databases (accessed in March 2019). All the parameters used with the assembly and annotation pipelines were the programmes' suggested defaults, unless otherwise stated. Only transcripts potentially

encoding proteins (based on ORF estimates or BLAST results) were retained in the final reference transcriptome. Assessments of assembly quality were obtained by running BUSCO (v. 3.1.0; Simão et al., 2015) against the Hymenoptera (odb9) database and rnaQUAST (v. 2; Bushmanova et al., 2016).

For the analyses of differential gene expression, reads from ants that experienced the HS and NHS treatments were aligned to the reference assembly using Salmon (v. 1; Patro et al., 2017). The transcripts that were differentially expressed between treatments were identified using edgeR (Robinson, McCarthy & Smyth, 2009) and DESeq2 (Love, Huber & Anders, 2014). Differential expression was considered to have occurred when transcripts displayed a mean absolute $\log_2$-fold change ≥ 2 between treatments and when FDR ≤ 1e-3 for both programs (edgeR and DESeq2). These two cut-offs are the recommended defaults for the Trinity pipeline. We computationally compared the list of differentially expressed genes between each pair of species using UniRef90 gene name annotation. Only unique and non-redundant genes (i.e., genes whose annotation did not contain the phrase “uncharacterised protein”) were included in this comparison and in the statistical tests. The significance of the number of differentially expressed genes shared between species was assessed using 10,000 random sampling iterations and an alpha level of 0.01 (R script available at https://github.com/nat2bee/ForagersvsNurses/blob/master/Statistics/common.stats.R). The final list of shared genes was then manually curated to avoid partial or redundant annotation matches.

To test whether gene ontology (GO) terms for biological processes (BPs) were enriched among the differentially expressed genes, we compared the latter’s BP annotations with the annotations of all the transcripts displaying non-zero expression in the treatment groups. We used edgeR and DESeq2 (*C. bombycina*: 29,965 transcripts; *C. cubica*; 31,029 transcripts; *C. piliscapa*: 26,176 transcripts; *C. holgerseni*: 26,769 transcripts; *C. mauritanica*: 24,741 transcripts; and *C. niger*: 29,345 transcripts) as well as the “weight01” algorithm with the Fisher enrichment test in the R package TopGO (Alexa & Rahnenfuhrer, 2016). Significance was evaluated based on an adjusted alpha level of 0.01.

Analysis of gene co-expression

To identify general changes in gene expression patterns across the six species, we performed a co-expression analysis of orthologous genes. To this end, we predicted the amino acid sequence and coding region of each transcript based on its open reading frame and using TransDecoder (v. 5.5.0; Haas et al., 2013). We obtained orthogroups among all six species using OrthoFinder (v. 3.3.12; Emms & Kelly, 2019). The replicates for the HS and NHS treatments were then aligned to the transcripts' coding regions using Bowtie2 (v. 2.4.2; Langmead & Salzberg, 2012). Estimates of gene expression levels—transcripts per million (TPM)—were obtained using RSEM (v. 1.3.3; Li & Dewey, 2011). Expression patterns for the orthogroups in which all the species were represented were then compared using Clust (v. 1.12.0; Abu-Jamous & Kelly, 2018). We were specifically interested in genes that were similarly co-expressed in response to the treatments (HS vs. NHS) across different species. We then verified the GO terms associated with BPs for the genes in each cluster using REVIGO (Supek et al., 2011). We assessed gene involvement in specific biochemical and metabolic pathways using the Kyoto Encyclopedia of Genes and Genomes (KEGG), via the KEGG Automatic Annotation Server (KAAS; Moriya et al., 2007).

Results

Heat tolerance and heat hardening

In the heat-stress experiments, percent survival showed a similar pattern across all six *Cataglyphis* species (Figure 3). Most workers tolerated temperatures of up to 43°C for three hours. Then, percent survival decreased significantly between 43°C and 45°C, depending on the species. CT_{max} was 43°C for *C. bombycina, C. piliscapa*, *C. mauritanica*, and *C. niger*; it was 45°C for *C. cubica* and *C. holgerseni.* Notably, percent survival for *C. cubica* and *C. holgerseni* was still higher than 90% at 45°C. No species could withstand temperatures above 47°C for the 3-hour exposure period.

Heat hardening significantly increased percent survival in *C. piliscapa*, *C. niger*, and *C. mauritanica* (Figure 4). These three *Cataglyphis* species live in regions with lower MaxT (Figure 2). In contrast, heat hardening decreased percent survival for *C. bombycina*, *C. holgerseni*, and *C. cubica*. Although this drop was only significant for *C. cubica*, the trend suggests there was a heat-loading effect for all three species. Stronger support for this interpretation comes from the Kaplan-Meier results, in which there was a significant heat-hardening effect for *C. piliscapa*, *C. niger*, and *C. mauritanica* as well as a significant heat-loading effect for *C. bombycina*, *C. holgerseni*, and *C. cubica* (Supplementary File 1, Figure S1).

Differential gene expression in response to heat stress

The sequenced samples and reference transcriptome assemblies for the six *Cataglyphis* species are available on the NCBI website (Bioproject PRJNA632584); the annotations for these assemblies are accessible at GitHub (https://github.com/nat2bee/Cataglyphis_HS/tree/main). The major quality parameters for the reference transcriptomes and the total number of transcripts in each assembly can be found in the supplementary materials (Supplementary File 1, Table S1). The number of transcripts differentially expressed between the HS and NHS treatments varied greatly across species,

ranging from 54 (*C. holgerseni*) to 1,118 (*C. niger*) (Supplementary File 1, Table S1). The list of all the transcripts that were differentially expressed in each species and their annotations are provided in the supplementary materials (Supplementary Files 2–13). The cross-species comparisons revealed a significant degree of overlap in the genes that were differentially expressed (Table 1). The differentially expressed genes shared by three or more species, as well as their expression patterns, can be found in Figure 5.

The number of enriched GO terms also varied among species: 4 in *C. bombycina*, 49 in *C. cubica*, 32 in *C. piliscapa*, 7 in C. *holgerseni*, 13 in *C. mauritanica*, and 16 in *C. niger* (Supplementary File 1, Table S2). “Protein refolding” (GO:0042026) was enriched in four species: *C. cubica*, *C. piliscapa*, *C. mauritanica*, and *C. niger*. It was marginally enriched in *C. bombycina* (Fisher $p = 0.025$), in whom several *hsp* genes were highly upregulated under heat stress (Figure 5). Other BP terms found to be frequently enriched in *Cataglyphis* were “cellular response to heat” (GO:0034605) and “chaperone cofactor-dependent protein refolding” (GO:0051085); such was notably the case in *C. piliscapa*, *C. mauritanica*, and *C. niger*. Additionally, the term “protein folding” (GO:0006457) was commonly enriched in *C. piliscapa* and *C. mauritanica,* as was the term “cellular response to unfolded protein” (GO:0034620) in *C. piliscapa* and *C. niger*. Overall, these results highlight the relevance of protein folding as one of the main molecular strategies used as an HSR in *Cataglyphis* ants. Moreover, for all six species, both the BP terms and the functions of the most differentially expressed genes fell into at least one of the following functional categories: cellular structural organisation, cellular signalling, transduction and transport, development, metabolism, and DNA/RNA regulation.

Co-expressed transcripts in response to heat stress

OrthoFinder assigned 283,560 proteins (92.5% of the total) to 39,565 orthogroups across the species studied; 10,786 of these orthogroups contained proteins found in all 6 species, which were used in the co-expression analysis. The co-expression analysis identified four clusters of orthogroups (C0, C1, C2, and C3) in which expression patterns were affected by the HS

treatment (Figure 6). Clust extracted these clusters because they were the largest groups of co-expressed genes with the least dispersion across samples (Abu-Jamous and Kelly, 2018). Therefore, in our analyses, the clusters represented a cohort of protein-encoding genes whose expression patterns varied in a correlated fashion across all six species between the heat-stress treatments. In these clusters, the number of orthogroups ranged from 138 (C1) to 240 (C0), and the number of genes ranged from 277 (C1, *C. mauritanica*) to 601 (C0, *C. niger*). The number of genes per species and the number of orthogroups in all the clusters are provided in the supplementary materials (Supplementary File 1, Table S3). The expression patterns of these clusters were the same in all six species, except in *C. mauritanica*, for which C1 and C3 showed the opposite patterns (Figure 6). In the HS treatment, in all six species, clusters C2 and C3 were upregulated (except in *C. mauritanica*, where C3 was downregulated), and clusters C0 and C1 were downregulated (except in *C. mauritanica*, where C1 was upregulated).

The complete functional annotations for the transcripts in each cluster can be found in the GitHub repository. Many genes commonly appeared in more than one cluster, such as genes encoding transcription factors, DNA helicase, DNA polymerase, cytochrome P450, proteins related to DNA repair, zinc-finger proteins, histone deacetylase, n-methyltransferase, ubiquitin protein ligase, actin, kinesin, myosin, ankyrin, chaoptin, cadherin, RAS signalling proteins, enzymes involved in inositol phosphate metabolism, and proteins involved in ionic balance regulation. The GO terms associated with the different clusters were also quite comparable (Supplementary File 1, Figures S2–S5). This finding indicates that active regulation of these pathways is a relatively conserved component of HSRs in the genus *Cataglyphis*.

There were, however, some unique features in each cluster. For example, the HSP-encoding genes *Hsp83, Hsp90*, and *bag family molecular chaperone regulator 3* were only seen in C2. Clusters also varied in gene diversity (i.e., within a gene family) and/or gene isoforms (i.e., of the same gene). For instance, although *e3 ubiquitin-protein ligase* genes occurred in all clusters, one isoform was found in C0, three were found in C1, two were found in C2, and nine were found in C3. A similar pattern was observed for the *cytochrome p450*

gene family, with one isoform in C0, five isoforms in C1, one isoform in C2, and no isoforms in C3. This result could explain the different expression patterns observed for *C. mauritanica* in C1 and C3.

To better visualise the metabolic and biochemical pathways that were co-regulated across species, we analysed the KEGG pathways represented in each cluster (Supplementary File 1, Figure S6). In all four clusters, there were genes involved in glutathione metabolism regulation (pathway: ko00480), either through the regulation of glutathione synthesis or its precursors (cysteine, glycine, and/or glutamate). Genes in clusters C0, C2 and C3 were involved in the regulation of fatty acid biosynthesis and elongation (M00083, M00084, M00085), as well as in the regulation of sphingosine and inositol phosphate metabolism. In C1, there were genes involved in the modulation of pentose phosphate metabolism (M00007) and of C10–20 isoprenoid biosynthesis (M00367). Finally, C0 and C2 contained genes associated with the regulation of galactose degradation (M00632); C1 and C2 contained genes associated with the regulation of spermine or spermidine biosynthesis (K00757 and K24034); and C3 contained genes associated with upregulation of nitric oxide synthesis (K13240).

Discussion

We characterised thermal tolerance and HSRs in six phylogenetically distant species of *Cataglyphis* ants. All the species are highly thermophilic, but they experience distinct thermal conditions as they occur in different types of habitats: arid sandy deserts, semi-arid mountainous regions, or Mediterranean coastal zones (Figure 2). Our results show that all six species displayed similar thermal tolerance levels and CT_{max} values. Furthermore, we identified many differentially expressed genes across all six species and four clusters of genes whose expression patterns were affected by heat stress. Interestingly, even when there were interspecific differences in the identities of these genes, the latter were involved in similar biological processes and molecular mechanisms, namely safeguarding the proteome, eliminating toxic residues, and preserving cellular integrity.

It was somewhat unexpected to find that species experiencing distinct thermal regimes displayed similar heat tolerance levels and CT_{max} values. Indeed, although workers of all six species typically leave the nest to forage at the hottest hours of the day, they experience very different thermal conditions; for example, maximum annual temperature is 29.0°C in the Mediterranean habitats of *C. piliscapa* and 42.3°C in the Saharan Desert habitats of *C. bombycina*. We also found great interspecific similarity in gene expression patterns in response to heat stress and in the biological processes associated with the differentially expressed and co-expressed genes. We posit that this shared transcriptomic response could underlie the similar thermal tolerance levels and CT_{max} values observed for the six species under laboratory conditions. These findings suggest that molecular responses to heat stress have been evolutionarily conserved in the ant genus *Cataglyphis*.

Our laboratory experiments allowed us to limit how much estimates of thermal tolerance were affected by confounding factors such as desiccation risk and/or morphological adaptations to heat (see below). Hence, our results highlight differences that are related exclusively to high temperatures; they cannot speak to other environmental challenges such as intense solar radiation. We predict that, under natural conditions, thermal tolerance and

370 CT_{max} should actually differ across *Cataglyphis* species because they each have unique
371 behavioural, morphological, and physiological adaptations that should lead to different levels
372 of heat-stress resistance (see the Introduction; Boulay et al., 2017; Perez & Aron, 2020). For
373 example, in nature, *C. bombycina* can use its prism-shaped hairs to reflect solar radiation via
374 total internal reflection, thus boosting its heat tolerance and limiting heat absorption when
375 exposed to direct sunlight (Shi et al., 2015; Willot et al., 2016). However, this remarkable
376 adaptive mechanism had no role to play in the context of our experiments.

377 We found that heat hardening increased heat tolerance in the three species (*C. piliscapa*,
378 *C. niger*, and *C. mauritanica*) found in habitats with lower maximum annual temperatures
379 (MaxT). In contrast, the three species that suffered from heat loading (*C. bombycina*, *C.*
380 *holgerseni*, and *C. cubica*) are those that endure higher maximum annual temperatures. This
381 result suggests a trade-off exists between the ability to tolerate extremely high temperatures
382 and the ability to enhance thermotolerance by means of heat hardening. These findings fit
383 with field observations showing that species that can more readily exploit heat hardening have
384 longer daily foraging windows (*C. piliscapa*, Cerdá et al., 1989; *C. mauritanica,* Knaden &
385 Wehner, 2005; *C. niger*, pers. obs.) than do species that can less readily exploit heat
386 hardening (*C. bombycina*, Wehner et al., 1992; *C. holgerseni* and *C. cubica,* pers. obs.).

387 We found that HSRs in *Cataglyphis* are quite versatile and involve a wide range of cellular
388 pathways and protective mechanisms. Such diverse responses to heat stress have rarely
389 been seen in insects (Zhang et al., 2015; Willot et al., 2018; Cui et al., 2019; Ma et al., 2019;
390 Tonione, Bi & TsuTsui, 2020). In *Cataglyphis*, it appears that heat tolerance may arise from
391 the regulatory dynamics of four major biological pathways (see the graphical synthesis in
392 Supplementary File 1, Figure S7): (*i*) the regulation of transcriptional machinery and DNA
393 metabolism, (*ii*) proteome safeguarding and restoration, (*iii*) the digestion and elimination of
394 toxic residues, and (*iv*) the preservation of cellular integrity. Each pathway will be addressed
395 in a paragraph below.

396 Many of the genes found to be associated with the HSRs are involved in transcription
397 (e.g., *nuclear protein 1, interferon regulatory factor 2 binding protein 2-like, and dna-directed*

398 *rna polymerase*); epigenome regulation (e.g., *histone h2a*, *histone h3*, *cysteine-rich protein 2-*
399 *binding protein*, *methyltransferase-like protein*, *nad-dependent deacetylase sirtuin 1,* and
400 *something about silencing protein 10*); and RNA or DNA metabolism (e.g., *rna-dependant*
401 *helicase*). Some of these genes are also involved in DNA preservation and repair. For
402 example, histone H2A and histone H3 may help safeguard and fix damaged DNA (Foster &
403 Downs, 2005; Bungard et al., 2010; Delaney et al., 2018). Additionally, DNA repair and
404 preservation are enhanced via the general regulation of *dna repair protein rad51 homolog* and
405 *dna repair protein xrcc4-like* and via the differential regulation of *nad-dependent deacetylase*
406 *sirtuin 1* (Mei et al., 2016) in *C. cubica*, *C. piliscapa*, and *C. niger*. Overall, the broad diversity
407 of genes associated with the regulation of transcription and DNA metabolism suggests that
408 the HSRs are rapid and transient, providing dynamic and efficient molecular protection.
409 We observed the differential expression of genes in all the *hsp* families (except Hsp100),
410 revealing that safeguarding and restoring the proteome is crucial during heat stress. The three
411 types of heat-shock proteins—Hsp70s, Hsp90s, and sHSPs—were all commonly expressed
412 in *Cataglyphis* (except in *C. holgerseni*). Among the sHSPs, protein lethal(2)essential for life
413 prevents the aggregation of misfolded proteins and ensures the protection of the actin
414 cytoskeleton (Mounier & Arrigo, 2002, Willot et al., 2018). Hsp70s and Hsp90s actively
415 participate in the protein-folding process; Hsp90s also stabilise the proteasome system
416 (Kimura et al., 1994; Evgen'ev et al., 2014). In ants, social bees, and social wasps, *hsp83*
417 (part of the Hsp90 family) and *hsc70-4* (part of the Hsp70 family) have a unique evolutionary
418 history—changes in cis-regulatory elements have given rise to various inducible forms
419 (Nguyen, Gotelli & Cahan, 2016). Consequently, it may be that highly inducible forms of *hsp90*
420 and *hsp70* were selected for in *Cataglyphis* in response to hot habitat conditions. Notably, the
421 upregulation of the transcription factor *interferon regulatory factor 2* decreases interferon
422 levels, resulting in an increase in Hsp70 levels (Kubo et al., 1996). This finding points to the
423 fundamental role of the Hsp70 family in HSRs. Other chaperones, like *dnaj* (Hsp40 homolog),
424 *PPIA* (peptidylprolyl cis-trans isomerase), and *bag family molecular chaperone* (Hsp70 co-

chaperone) were significantly co-regulated in all six species, indicating the presence of diverse mechanisms for safeguarding the proteome.

Part of proteostasis is the digestion and elimination of toxic residues, such as protein aggregates and ROSs. Here, we found that heat stress induces the upregulation of genes involved in the ubiquitin proteasome system (UPS), like *ZFAND*, *E3 ubiquitin ligase*, *rbr type E3 ubiquitin transferase*, and *protein roadkill*, which contribute to protein degradation (van den Heuvel, 2004; Park et al., 2007). The elimination of protein aggregates is also promoted by the lysosomal system and through autophagy (Ihara, Kawashima & Nixon, 2012; Kumsta et al., 2017). Lysosomal functions are regulated by nitric oxide synthesis (Li et al., 2016), and we observed genes involved in KEGG pathways related to nitric oxide synthesis. In contrast, only a few differentially-expressed transcripts appeared to be directly involved in autophagy: *VPS13D* in *C. bombycina* and *sequestosome-1* in *C. mauritanica*. Our analyses also confirmed the presence of genes involved in KEGG pathways related to the regulation of spermine and spermidine biosynthesis. It is thought that spermidine is involved in autophagy (Minois et al., 2014); it has been found that this compound works with spermine to improve thermal tolerance (Sagor et al., 2013). In the six *Cataglyphis* species, ROS detoxification seems to be supported by the regulation of genes involved in the production of glutathione (or its precursors) and the regeneration of NADPH, as well as by the regulation of several genes encoding cytochrome P450. Increases in glutathione production and NADPH regeneration via the pentose phosphate pathway can enhance ROS elimination, which relies on the activity of antioxidants like glutathione reductase and members of the cytochrome P450 family (CYP450) (Birben et al., 2012; Mullarky & Cantley, 2015). The role of CYP450 in the antioxidant response has been seen in many organisms, including in plants and animals (e.g., Xing et al., 2013; Ma et al., 2019; Pandian et al., 2020).

Cataglyphis workers appear to preserve cellular integrity through membrane modification and cytoskeletal rearrangement. We observed the involvement of genes encoding lipases (*lysophospholipase D GDPD1, phospholipase ddhd1, lipase*) and compounds involved in phospholipid synthesis (via the metabolism of inositol phosphate [e.g., *protein Opi10 homolog,*

453 *phosphatidylinositol 3-kinase*, and *inositol polyphosphate 5-phosphatase*] and sphingosine
454 [e.g., *sphingosine kinase*]), which are known to help modify cell membrane composition
455 (Britanica, 2009; Patel et al., 2019). Moreover, we saw genes involved in lipid elongation and
456 isoprenoid production (i.e., of sterols). Membranes containing sterols and larger proportions
457 of long-chain phospholipids are less fluid under hot conditions (Quinn, 1988; Nozawa, 2011;
458 Dufourc, 2008). These results are consistent with the idea that HSRs lead to cell-membrane
459 stiffening in *Cataglyphis* ants. Cytoskeletal rearrangement necessitates the regulation of
460 cytoskeletal elements and of signalling molecules. We observed the upregulation of genes
461 encoding cytoskeletal proteins like actin, kinesin, myosin, and tropomyosin. We also saw the
462 upregulation of genes encoding anchoring proteins, such as cadherin and chaoptin, which
463 promote cell adhesion (Krantz & Zipursky, 1990; Angst, Marcozzi & Magee, 2001). The
464 upregulation of myosin and tropomyosin production indicates that a need exists for protecting
465 muscular function, so that *Cataglyphis* workers can maintain high running speeds during
466 foraging (Willot et al., 2018). Finally, our results showed that genes encoding RAS signalling
467 molecules (e.g., *ras gtpase*, *rho gtpase,* and *ras related proteins*) were being regulated; these
468 compounds are involved in intracellular trafficking and organelle stabilisation (Etienne-
469 Manneville, 2002). Working together, these mechanisms enhance cellular stability and
470 optimise cellular functioning.

471 In conclusion, we discovered that thermal tolerance and HSRs are similar across
472 phylogenetically distant species of *Cataglyphis* ants, despite the differences in the thermal
473 regimes they experience in nature. Their high levels of thermal tolerance mechanistically arise
474 from a diversity of biological processes, including the regulation of genes involved in
475 transcription and DNA metabolism, proteome stability, the elimination of toxic residues, and
476 the maintenance of cellular integrity via membrane modifications and cytoskeletal
477 rearrangements. This suite of molecular HSRs allow *Cataglyphis* ants to forage even when
478 temperatures outside are close to the ants' physiological limits. Further research should
479 examine whether such cellular and molecular adaptations have convergently evolved in other
480 highly thermophilic ant species.

Acknowledgments

This work was supported by the Belgian *Fonds National pour la Recherche Scientifique* (grant numbers J.0151.16 and T.0140.18 to SA and PhD grant to RP). We are grateful to Morocco's High Commission for Water, Forests and Combatting Desertification (HCEFLCD; Decision 11/2018 and 23/2019) for granting us collection permits. We thank A. Kuhn, N. Lecocq de Pletincx and Q. Willot for their help with field collections, and J. Pearce-Duvet for her language editing services.

Conflict of interest

The authors declare no conflict of interest.

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Data Accessibility and Benefit-Sharing Statement

RNAseq data and transcriptome assemblies are available at NCBI under the Bioproject PRJNA632584.
Transcriptomes full annotation and Clusters per species are available at the manuscript public repository at GiHub https://github.com/nat2bee/Cataglyphis_HS.

Author Contributions

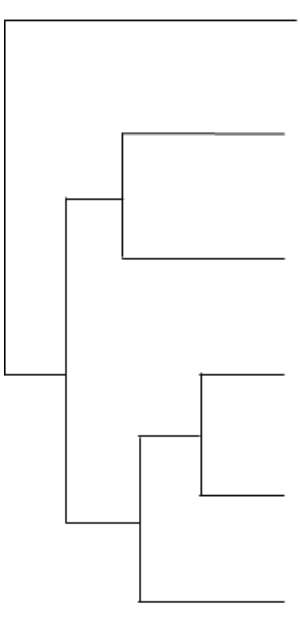
R.P. and S.A. conceived the study and collected samples. N.S.A. and M.D. designed computer analysis which were performed by N.S.A.
R.P. and N.S.A. analyzed the data. All authors contributed to drafting the article, approved the final published version and agreed to be held accountable for all aspects of the work

Table 1. Number of differentially expressed genes among the six *Cataglyphis* ant species.

The number of differentially expressed genes shared by each species pair is shown in the upper right-hand part of the table. Shown in the lower left-hand part of the table are the mean numbers (± standard deviation) of the differentially expressed genes expected to be shared by each species pair, which were estimated based on the distributions of 10,000 random iterations. The cells in grey show the numbers of unique non-redundant genes that were differentially expressed in each species. * = the species shared a significant number of genes ($p < 0.01$).

	C. piliscapa	*C. mauritanica*	*C. bombycina*	*C. holgerseni*	*C. niger*	*C. cubica*
C. piliscapa	62	8*	15*	1	16*	12*
C. mauritanica	1.62 ± 1.27	152	16*	4*	43*	15*
C. bombycina	1.19 ± 1.09	3.14 ± 1.74	113	2	31*	20*
C. holgerseni	0.44 ± 0.65	1.16 ± 1.07	0.85 ± 0.92	40	3	5
C. niger	4.99 ± 2.15	12.49 ± 3.4	9.19 ± 2.95	3.42 ± 1.81	503	59*
C. cubica	4.86 ± 2.15	10.51 ± 3.13	7.72 ± 2.69	2.82 ± 1.64	32.31 ± 5.43	437

Figure 1. *Cataglyphis holgerseni* worker in the Neguev desert. © Alexandre Kuhn



	AMT	MDR	MTWQ	MaxT	MTDQ
C. piliscapa	15.5	9.0	22.7	29.0	22.7
C. mauritanica	10.4	14.4	19.2	30.5	19.2
C. bombycina	21.0	17.2	30.8	42.3	30.8
C. holgerseni	22.9	13.1	29.6	37.3	29.6
C. niger	20.2	10.6	26.2	32.0	25.6
C. cubica	17.7	16.5	25.5	36.9	25.4

Figure 2. Phylogenic relationships among the six *Cataglyphis* species based on the transcriptome data (as estimated via OrthoFinder).

This tree is consistent with those previously established for the same genus (Aron et al. 2016; Kuhn et al. 2019). The main thermal characteristics of the habitats are given for each species. AMT = annual mean temperature; MDR = mean diurnal range; MTWQ = mean temperature of the warmest quarter of the year; MaxT = highest temperature of the warmest month recorded; and MTDQ = mean temperature of the driest quarter. All values are in °C. The climatic data were obtained from the WorldClim database using a resolution of 30 arc-seconds.

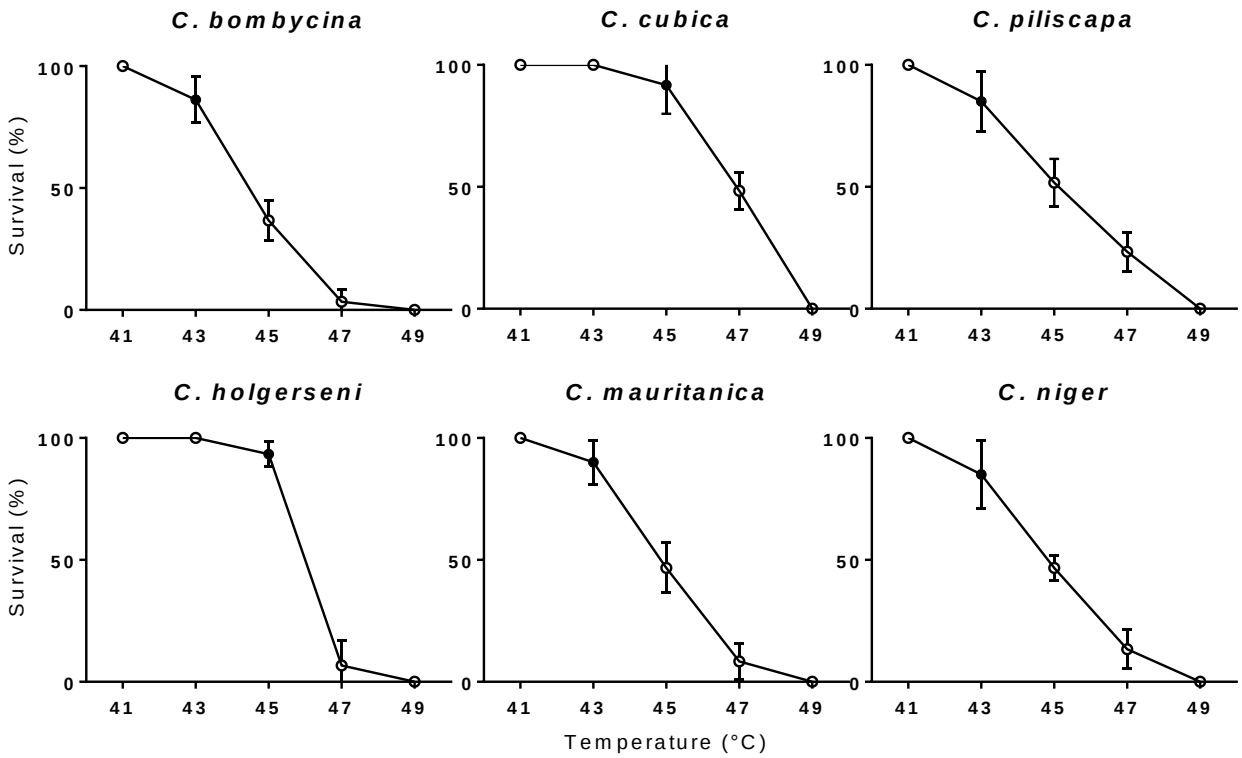


Figure 3. Percent survival of *Cataglyphis* workers in the heat-stress treatment.

The ants were exposed to five temperatures (heat baths kept at 41°C, 43°C, 45°C, 47°C, or 49°C) for 3 hours; their percent survival was then measured. For each species, CT_{max} is indicated by the filled circle (•).

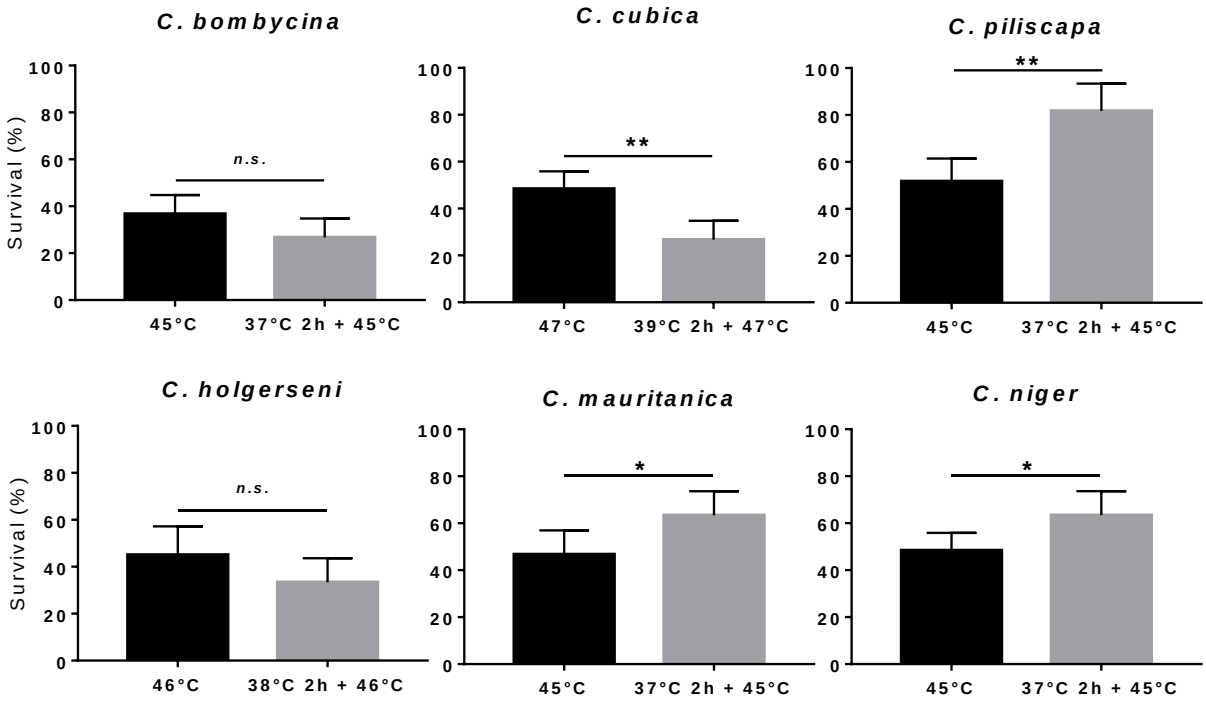


Figure 4. Percent survival of heat-hardened or non-heat-hardened *Cataglyphis* workers exposed to heat stress.

Percent survival was measured after the ants were exposed to T_{50} for 3 hours; one group had experienced heat hardening (preheating at 8°C below T_{50} for 2 hours; grey bars), and the other group had not (no preheating treatment; black bars). The temperatures used in each experiment for each species are shown. Student *t*-tests: *n.s.*: $p > 0.05$, *: $p < 0.05$, **: $p < 0.01$.

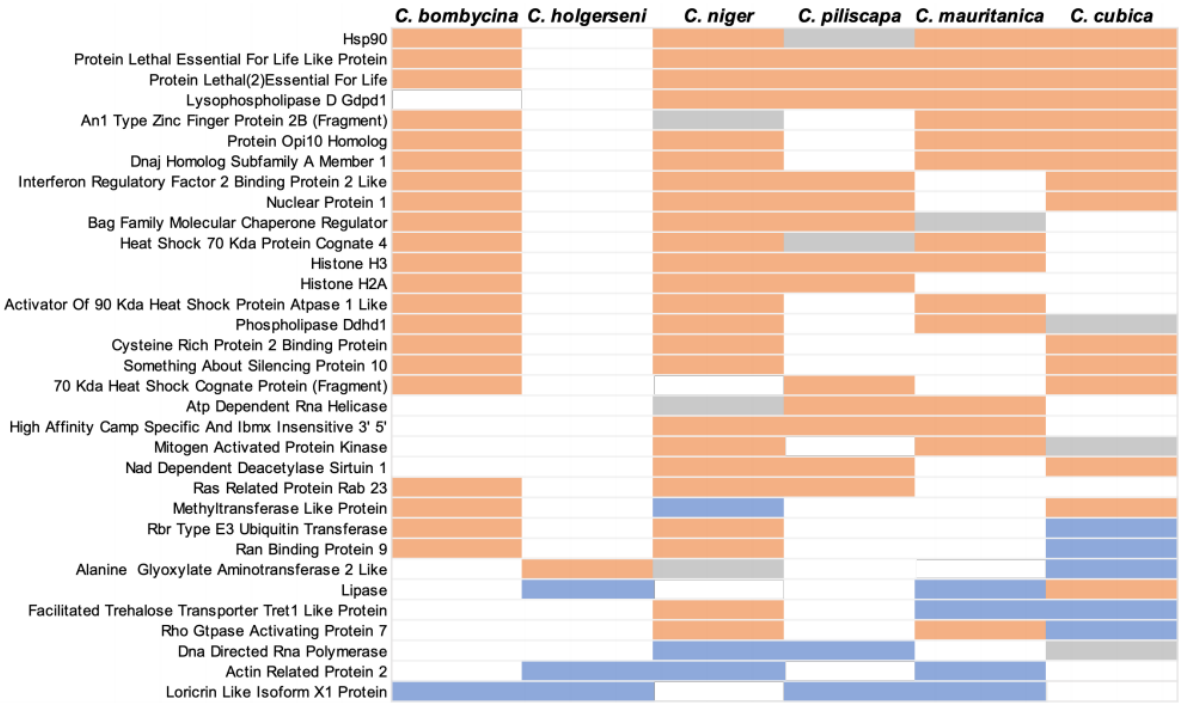


Figure 5. Differentially expressed genes (mean $\log_2$-fold change ≥ 2 and FDR ≤ $1e^{-3}$) that were shared by three or more *Cataglyphis* species in response to heat stress.

The colour indicates the specific gene expression pattern; salmon: genes with greater expression in the heat-stress (HS) treatment; blue: genes with higher expression in the no-heat-stress (NHS) treatment; grey: pattern of upregulation is the same in both the HS and NHS treatment.

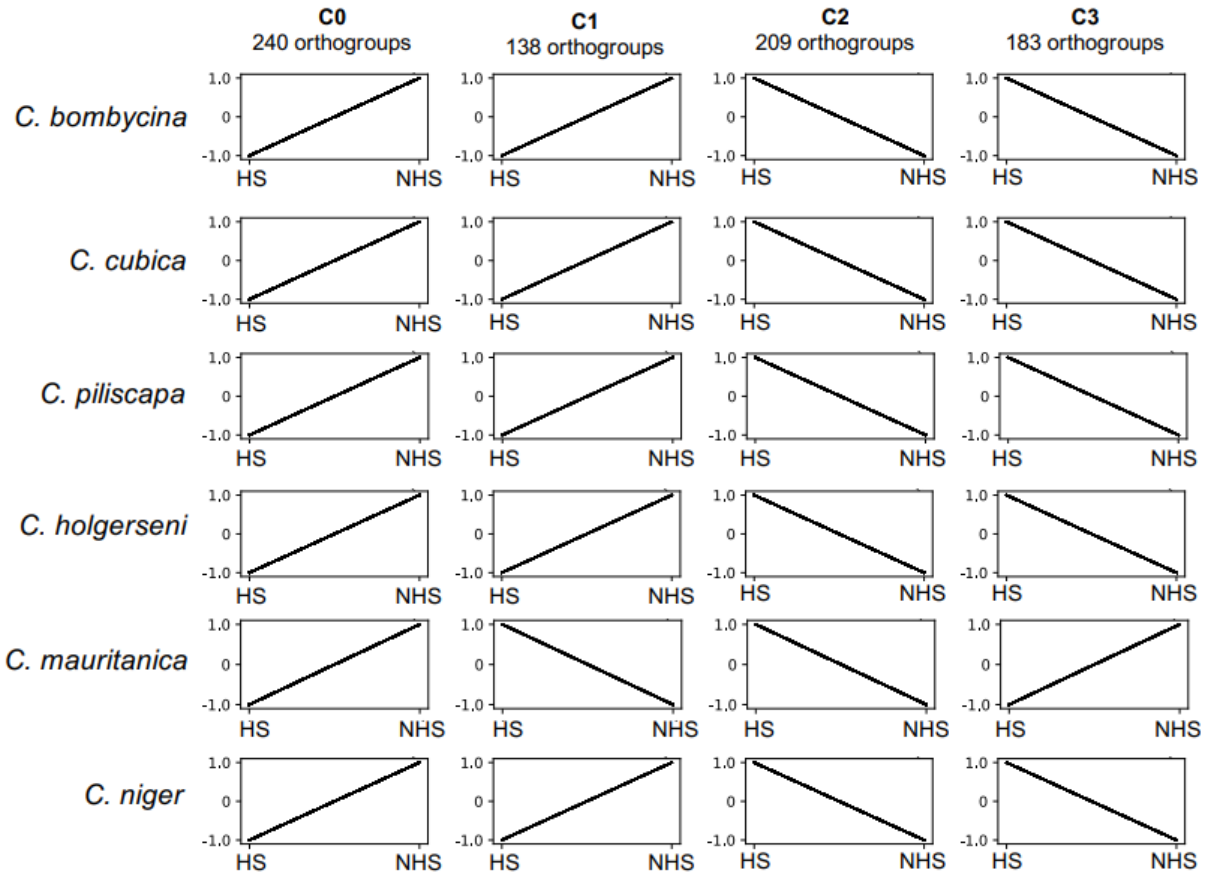


Figure 6. Gene expression patterns in *Cataglyphis* workers exposed to heat stress for the orthogroups in each cluster.

The y-axis indicates the cluster-level z-scores for expression as estimated in Clust using transcripts per million as a metric. The median expression values for the orthogroups for all four replicates per species are shown.

Additional Files

Supplementary File 1: Kaplan-Meier curves, major summary parameters of transcriptomic analysis, GO enrichment results, Co-expression analysis and graphical abstract of the heat-shock response.

Supplementary File 2–13: The list of all the transcripts that were differentially expressed in each species and their annotations.