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Molecular adaptations to heat stress in the thermophilic ant genus *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 *Cataglyphis* species from distinct phylogenetic groups that live in different habitats and experience different thermal regimes. We found that all six species had high heat tolerance levels with critical thermal maxima (CT_{max}) ranging from 43°C to 45°C and a median lethal temperature (LT50) ranging from 44.5°C to 46.8°C. Transcriptome analyses revealed that, although the number of differentially expressed genes varied widely for the six species (from 54 to 1118), many were also shared. Functional annotation of the differentially expressed and co-expressed genes 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 diversity may help workers withstand temperatures close to their physiological limits.

KEYWORDS
ants, CT_{max}, heat stress, molecular adaptation, RNA-seq, thermotolerance

1 | INTRODUCTION

Thermal stress is a daunting physiological challenge for living organisms (Evgen'ev et al. 2014). Extreme temperature variation disturbs the thermodynamic equilibria of chemical bonds and alters the three-dimensional structure of macromolecules, leading to their denaturation (Feder and Hofmann 1999; Quinn, 1988; Wang et al., 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 (Birben et al., 2012; Evgen'ev et al. 2014; Hazel, 1995). 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 et al., 2010). In addition to their housekeeping role, HSPs perform several essential functions during heat stress: they ensure the correct folding of other

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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 ROS can be dampened via the production of antioxidants and detoxification enzymes, such as superoxide 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 Heikkila, 2017; Lockwood et al., 2015; Logan & Buckley, 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. Yet, the study of thermophilic species—adapted to withstand and thrive under high thermal stress—is essential to understand the mechanisms of thermal adaptation.

In this study, we examined molecular adaptations to heat stress in one of the most heat-tolerant animals described to date: *Cataglyphis* desert ants. This ant genus 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 (Gehring and Wehner 1995; Cerdá et al., 1998; Wehner et al., 1992). 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, such as *C. piliscapa* (previously *C. cursor*), have workers that 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 and Wehner 2012; Pfeffer et al., 2019). *C. 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 and Wehner 1995; Willot et al., 2017, 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 organization of sarcomeres (*unc-89*) and the safeguarding of cytoskeletal fibres via the action of small HSPs (sHSPs), which reveals the importance of maintaining proper motor function, as the ants forage at high speeds on the burning ground.

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

Since high thermal tolerance is widespread within *Cataglyphis*, this genus represents a rare model to study heat adaptations through an evolutionary perspective. Herein, we compared heat tolerance and gene expression patterns across six *Cataglyphis* species, from phylogenetically distinct groups (see File S1: Figure S1), that live in different habitats and experience different thermal regimes (Figure 2). First, we explored heat tolerance by measuring worker survival under conditions of heat stress, and investigated the ability of workers to acquire thermotolerance via heat hardening. Second, we examined the differential expression and co-expression of genes for control versus heat-stressed workers across species. Finally, through the comparative analyses of these results, we searched to differentiate more recent species-specific from conserved response mechanisms across *Cataglyphis* species. Our study aims to explore the molecular mechanisms of the heat stress response in these highly thermophilic ants, and to provide a better understanding about their physiomolecular responses in the face of extreme thermal events.

2 | MATERIALS AND METHODS

2.1 | Ant sampling and rearing conditions

We studied six *Cataglyphis* 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)

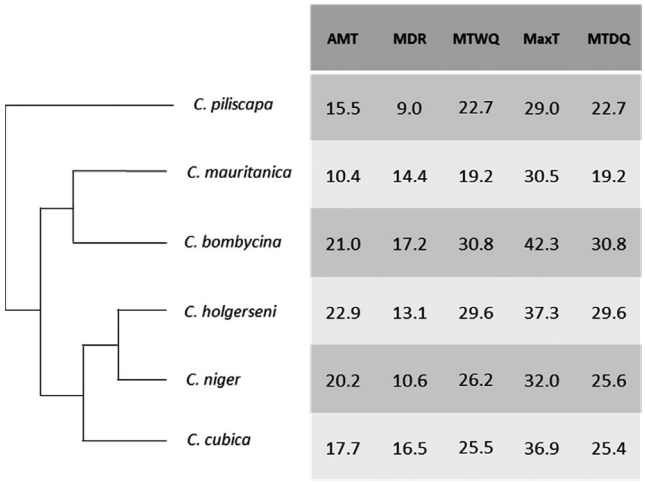

FIGURE 2 Phylogenetic relationships among the six *Cataglyphis* species based on the transcriptome data (as estimated via ORTHOFINDER). This tree is consistent with those previously established for the same genus (Aron et al. 2016; Kuhn et al. 2019). The main thermal characteristics of the habitats are 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

and *C. cubica* (Morocco: 32°00′04″N, 6°43'30''W); 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, annual mean 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 four to six colonies of each species and brought them back to the laboratory. 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 1 month before the experiments began.

2.2 | Assessing heat tolerance and heat-hardening capacity

Ant heat tolerance was characterized using a heat-stress experiment. For each species, six groups of 10 randomly selected workers were formed; these groups were then placed in glass tubes. Because body size influences heat tolerance due to differences in relative water loss (Buxton et al., 2021; Cerdá & Retana, 2000; Clémencet et al., 2010; Hood & Tschinkel, 1990), a wet cotton ball was added in the testing tubes to prevent ant desiccation. The glass tubes were immersed in a digitally controlled water bath kept at a constant temperature (SW22, Julabo GmbH). Heat-stress (HS) trials were conducted using five different temperatures: 41, 43, 45, 47 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) connected to a digital thermometer (RS Pro RS52 Digital Thermometer, RS Components Ltd). Per cent survival after 3 h was recorded; workers were classified as dead once they lost their locomotor ability (i.e., muscular paralysis). Previous studies have shown that 3 h 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 determined two estimates of the heat tolerance: (i) the critical thermal maximum (CT_{max}), the temperature below which the survival rate is significantly different from 100% (ANOVA test followed by a Tukey's test; $p < .05$), and (ii) the median lethal temperature (LT50), the temperature at which survival probability equals 50%. LT50 was estimated from a simple logistic regression of death probability as a function of temperature. The LT50 and 95% confidence intervals were compared between species using the ratio test (Wheeler et al. 2006), implemented in the *ratio_test* function from the ECOTOX package (Hlina, 2019).

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 per cent survival was ~50% (T_{50}). In the heat-hardening experiments, workers were exposed to a temperature that was 8°C below T_{50} for 2 h. 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 h. The mean per cent survival of heat-hardened workers (2 h at T_{50} minus 8°C + 3 h at T_{50}) and nonheat-hardened workers (3 h 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 (version 4.0; R Development Core Team, 2017).

2.3 | RNA-sequencing library preparation and sequencing

RNA extraction, RNA quality (determined via Bioanalyzer) and RNA-sequencing (RNA-seq) library preparation and sequencing were performed by BGI Tech Solutions. Total RNA was extracted from whole ant bodies using Trizol (Invitrogen) in accordance with the manufacturer's instructions. For each species, we analysed patterns of differential gene expression using four replicates per treatment; HS = workers at CT_{max} for 3 h versus NHS = workers at 25°C for 3 h. Heat-stressed and nonheat stressed ants were immediately frozen after treatment and stored at −80°C until RNA extraction. 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 replicate. 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.

2.4 | Transcriptome assembly and analyses of differential 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 (version 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 normalized the reads (20× coverage). Second, we assembled the reads using the TRINITY pipeline (version 2.8.4; Grabherr et al., 2011) and two independent strategies: full *de novo* assembly and reference-guided assembly. The reference-guided approach utilized the genome of *Cataglyphis hispanica* (unpublished data). These two independently assembled transcriptomes were then merged, and redundant transcripts were removed using CD-HIT (version 4.8.1; Huang et al., 2010), applying a threshold of 95% nucleotide similarity. Finally, transcripts were clustered into superTranscripts via CORSET (version 1.08; Davidson & Oshlack, 2014) and LACE (version 1.13; Davidson et al., 2017), which further reduced transcript redundancy and improved posterior gene expression counts (Davidson et al., 2017). This assembly was then annotated using ANNOCRIPT (version 2.0.1; Musacchia et al., 2015) in tandem with the UniProt Reference Cluster (UniRef90) and UniProtKB/Swiss-Prot (Suzek et al., 2015; The UniProt Consortium, 2021) databases (accessed in March 2019). Only transcripts potentially encoding proteins (based on open reading frame [ORF] estimates or BLAST results) were retained in the final reference transcriptome. All the parameters used with the assembly and annotation pipelines were the programs' suggested defaults, unless otherwise stated. Assessments of assembly quality were obtained by running BUSCO (version 3.1.0; Simão et al., 2015) against the Hymenoptera (odb9) database and RNAQUAST (version 2; Bushmanova et al., 2016).

For the analyses of differential transcript expression, reads from ants that experienced the HS and NHS treatments were aligned to the reference assembly using SALMON (version 1; Patro et al., 2017). The transcripts that were differentially expressed between treatments were identified using EDGER (Robinson et al., 2009) and DESEQ2 (Love et al. 2014). Differential expression was considered to have occurred when transcripts displayed a mean absolute $\log_2$-fold change ≥2 between treatments and false discovery rate (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 transcripts between each pair of species using UniRef90 gene name annotation. Only unique and nonredundant genes (i.e., genes whose annotation did not contain the phrase "uncharacterized 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 .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 transcripts, we compared the latter's BP annotations with the annotations of all the transcripts displaying nonzero expression in the treatment groups (*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). We used the "weight01" algorithm with the Fisher enrichment test in the R package TOPGO (Alexa & Rahnenfuhrer, 2016) for the enrichment test, and significance was evaluated based on an adjusted alpha level of .01.

2.5 | Analysis of gene co-expression

To identify general changes in gene expression patterns across the six species, we performed a co-expression analysis of orthologues. To this end, we predicted the amino acid sequence and coding region of each transcript based on its ORF using TRANSDECODER (version 5.5.0; Haas et al., 2013). We obtained orthogroups among all six species using ORTHOFINDER (version 3.3.12; Emms & Kelly, 2019) through its standard DEndroBLAST workflow, which uses STAG for species tree inference (Emms & Kelly, 2018). The replicates for the HS and NHS treatments were then aligned to all proteins using BOWTIE2 (version 2.4.2; Langmead & Salzberg, 2012). Estimates of the coding transcripts expression levels—transcripts per million (TPM)—were obtained using RSEM (version 1.3.3; Li & Dewey, 2011). Expression counts per protein along with orthogroups protein mapping information were used for the co-expression analyses with CLUST (version 1.12.0; Abu-Jamous & Kelly, 2018). Only orthogroups containing proteins from all species were considered in the co-expression analysis. 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).

3 | RESULTS

3.1 | Heat tolerance and heat hardening

In the heat-stress experiments, most workers tolerated temperatures of up to 43°C for 3 h (Figure 3). Then, per cent survival decreased significantly between 43 and 45°C, depending on the species. CT_{max}

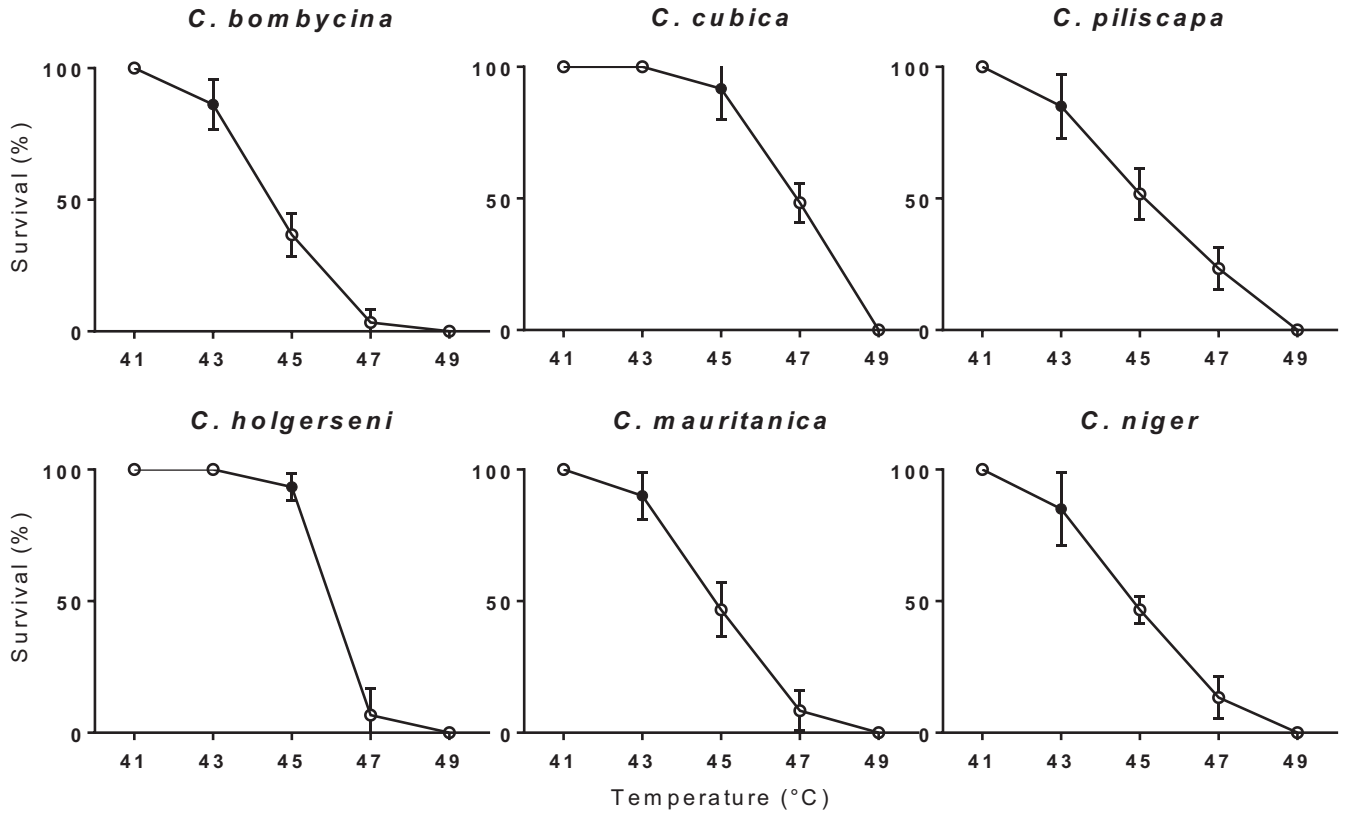

FIGURE 3 Per cent survival of *Cataglyphis* workers in the heat-stress treatment. The ants were exposed to five temperatures (heat baths kept at 41°C, 43°C, 45°C, 47°C or 49°C) for 3 h; their p ercent survival was then measured. For each species, CT_{max} is indicated by the filled circle (•)

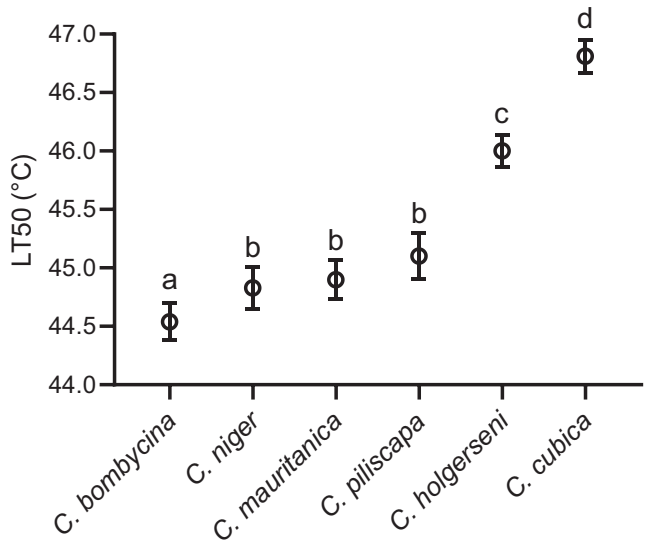

FIGURE 4 Median lethal temperature (LT50) of *Cataglyphis* workers in the heat-stress treatment. LT50 and 95% confidence intervals were calculated from a simple logistic regression of death probability. A ratio test was used for pairwise comparisons between species. Letters denote significantly different values ($p < .05$)

was 43°C for *Cataglyphis bombycina*, *C. niger*, *C. mauritanica* and *C. piliscapa* (comparison of survival rate using Tukey's test; 41°C versus 43°C: $p > .05$ and 43°C versus 45°C: $p < .001$ for all species); it was 45°C for *C. holgerseni* and *C. cubica* (43°C versus 45°C: $p > .05$ and 45°C versus 47°C: $p < .001$ for both species). Notably, per cent survival for *C. holgerseni* and *C. cubica* was still higher than 90% at 45°C. No species could withstand temperatures above 47°C for the 3-h exposure period. LT50 differed significantly among species (Figure 4). It was 44.54°C (95% confidence interval [CI]: 0.16) for *C. bombycina*, 44.83°C (0.18) for *C. niger*, 44.9°C (0.16) for *C. mauritanica* and 45.1°C (0.19) for *C. piliscapa*. The highest LT50 values were obtained for *C. holgerseni* and *C. cubica*, with 46°C (0.14) and 46.81°C (0.14) respectively. These values were in line with the CT_{max}, and show that *C. holgerseni* and *C. cubica* are the most heat-tolerant species.

Heat hardening significantly increased per cent survival in *C. niger*, *C. mauritanica* and *C. piliscapa* (Figure 5). These three *Cataglyphis* species live in regions with lower MaxT (Figure 2). In contrast, heat hardening decreased per cent 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

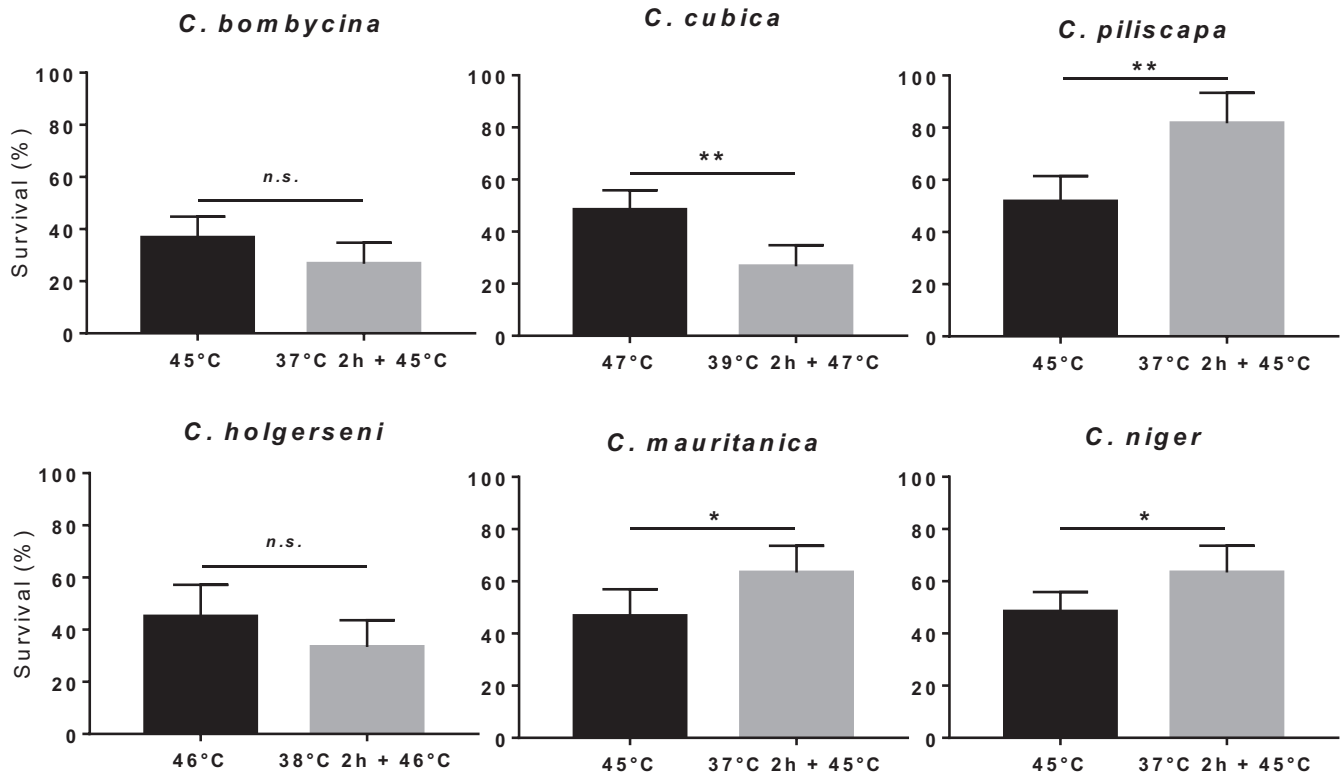

FIGURE 5 Per cent survival of heat-hardened or nonheat-hardened *Cataglyphis* workers exposed to heat stress. Per cent survival was measured after the ants were exposed to T_{50} for 3 h; one group had experienced heat hardening (preheating at 8°C below T_{50} for 2 h; grey bars), and the other group had not (no preheating treatment; black bars). The temperatures used in each experiment for each species are shown. Student *t*-tests: *n.s.*: $p > .05$, *: $p < .05$, **: $p < .01$

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

	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

Notes: 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 (±*SD*) 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 nonredundant genes that were differentially expressed in each species. *The species shared a significant number of genes ($p < .01$).

the Kaplan–Meier results, in which there was a significant heat-hardening effect for *C. niger*, *C. mauritanica* and *C. piliscapa* as well as a significant heat-loading effect for *C. bombycina*, *C. holgerseni* and *C. cubica* (File S1:Figure S2).

3.2 | Differential 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 File S1 and 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*) (File S1:Table S1). The list of all the transcripts that were differentially expressed in each species and their annotations are provided in Files S2–S13. The cross-species comparisons revealed a significant degree of overlap in the genes that were differentially expressed for most species

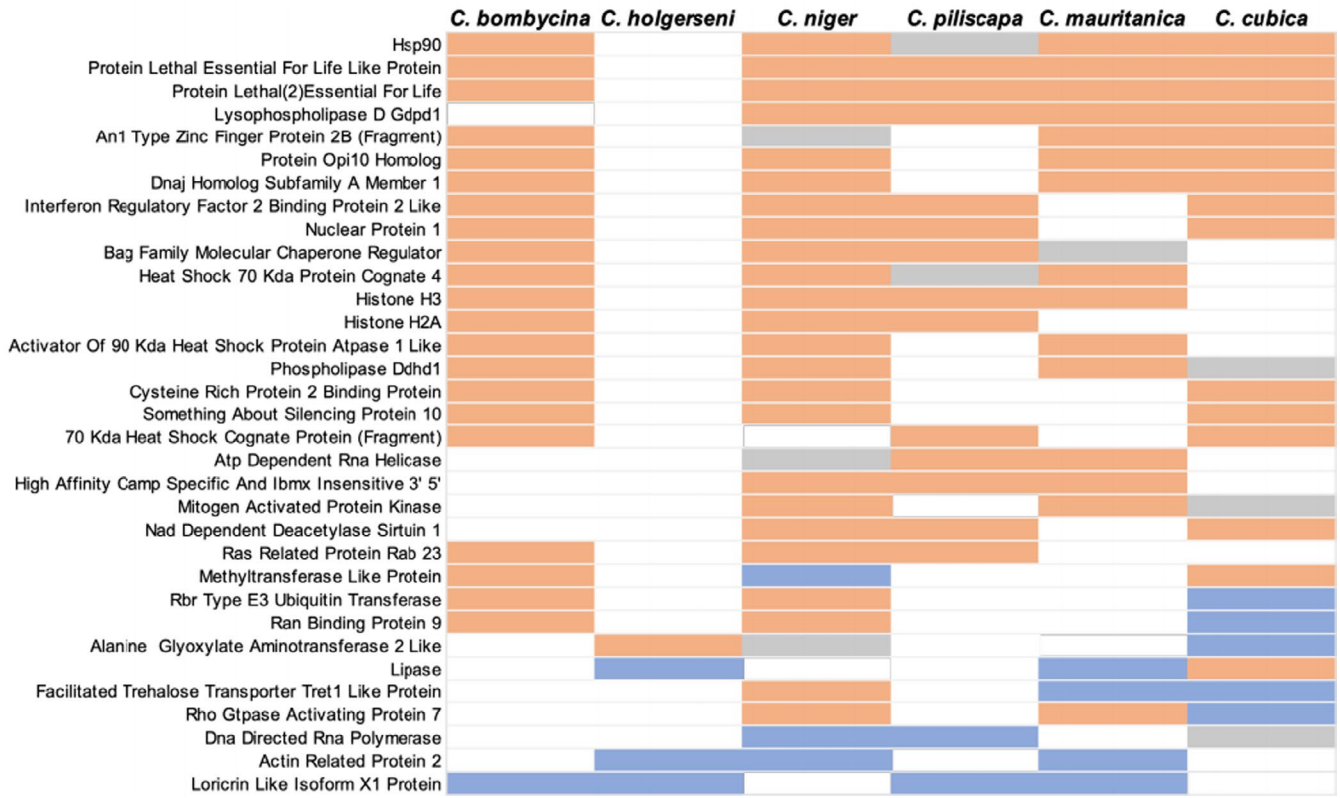

FIGURE 6 Differentially expressed genes (mean $\log_2$-fold change ≥2 and FDR ≤$1e^{-3}$) that were shared by three or more *Cataglyphis* species in response to heat stress. The colour indicates the specific gene expression pattern; salmon: genes with greater expression in the heat-stress (HS) treatment; 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

(Table 1). The differentially expressed genes shared by three or more species, as well as their expression patterns, are given in Figure 6.

The number of enriched GO terms also varied among species: four in *C. bombycina*, 49 in *C. cubica*, 32 in *C. piliscapa*, seven in *C. holgerseni*, 13 in *C. mauritanica* and 16 in *C. niger* (File S1: Table S2). "Protein refolding" (GO:0042026) was enriched in four species: *C. cubica*, *C. piliscapa*, *C. mauritanic*, and *C. niger* (Fisher $p < .01$). In *C. bombycina*, several *hsp* genes were highly upregulated (Figure 6); nevertheless, "protein refolding" was not significantly enriched according to our *p*-value cut-off at <.01 (Fisher $p = .025$). 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 organization, cellular signalling, transduction and transport, development, metabolism, and DNA/RNA regulation.

3.3 | 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 six 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 7). CLUST extracted these clusters because they were the largest groups of co-expressed genes with the least dispersion across samples (Abu-Jamous & 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 File S1 and 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 7). 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).

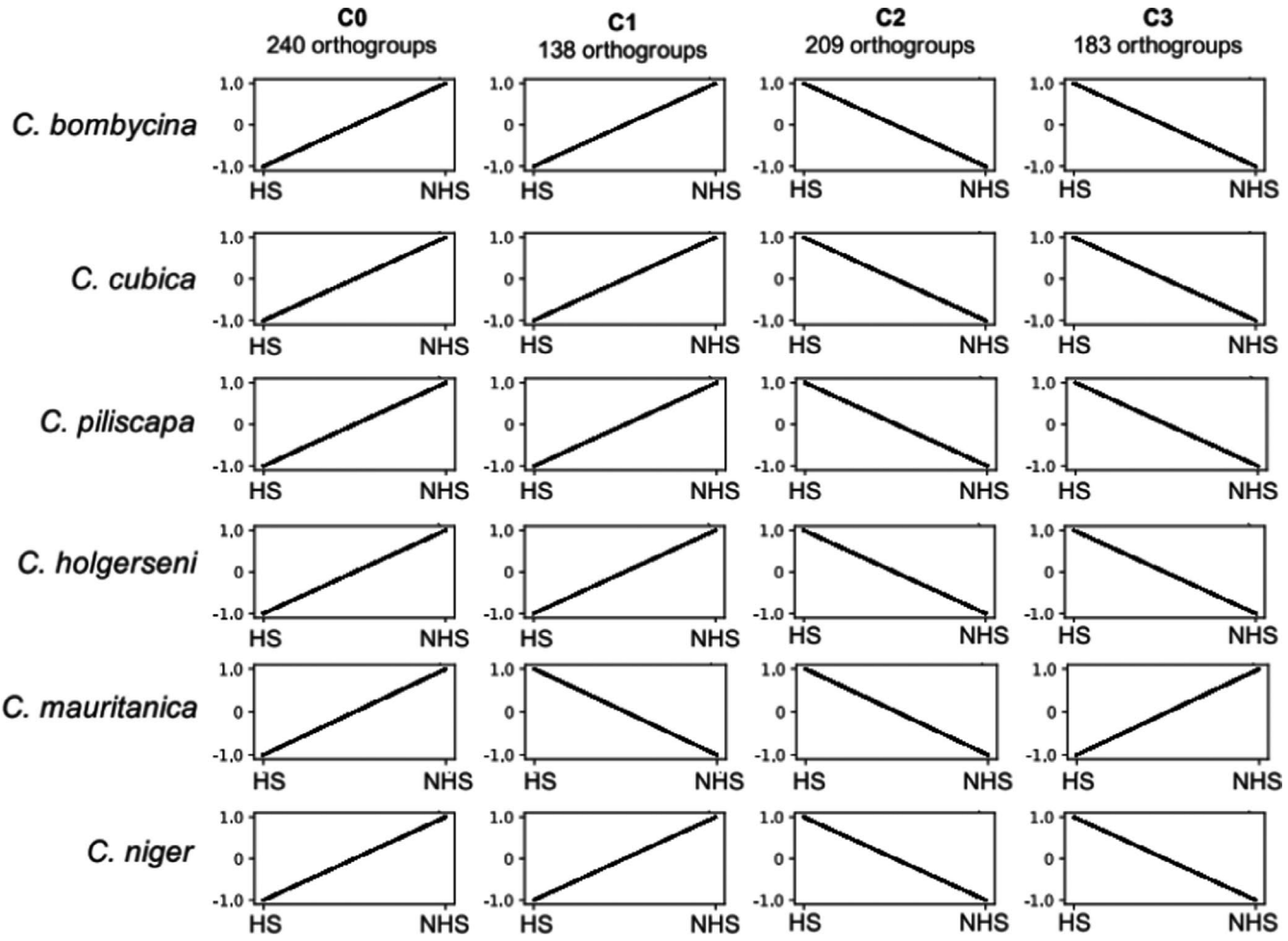

FIGURE 7 Gene expression patterns in *Cataglyphis* workers exposed to heat stress for the orthogroups in each cluster. The *y*-axis indicates the cluster-level z-scores for expression as estimated in CLUST using transcripts per million as a metric. The median expression values for the orthogroups for all four replicates per species are shown

The complete functional annotations for the transcripts in each cluster can be found in the GitHub repository. Some gene annotation terms (probably representing genes from the same family or multiple isoforms from the same gene in our transcriptomes) 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 (File S1: Figures S3–S6). 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 to 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 visualize the metabolic and biochemical pathways that were coregulated across species, we analysed the KEGG pathways represented in each cluster (File S1: Figure S7). 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).

4 | DISCUSSION

We characterized thermal tolerance and HSRs in six *Cataglyphis* species from different phylogenetic groups. 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 high thermal tolerance levels with a CT_{max} ranging from 43 to 45°C and a LT50 from 44.5 to 46.8°C. Furthermore, we identified many differentially expressed transcripts 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. Despite substantial interspecific differences in the number of differentially expressed transcripts (e.g., 54 for *C. holgerseni* and 1118 for *C. niger*) and, to a lesser extent, in CT_{max} and LT50, we found great 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 high thermal tolerance levels observed for the six species under laboratory conditions. These findings suggest that molecular responses to heat stress could be evolutionarily conserved in the ant genus *Cataglyphis*.

The finding that species experiencing distinct thermal regimes under natural conditions all displayed high heat tolerance levels was somewhat unexpected. 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 (Figure 2); for example, maximum annual temperature is 29.0°C in the Mediterranean habitats of *C. piliscapa* but 42.3°C in the Saharan Desert habitats of *C. bombycina*. Despite a high tolerance to heat stress, our quantitative approach based on LT50 still reveals some differences among the species studied, with *C. cubica* and *C. holgerseni* being significantly more heat-tolerant. Surprisingly, *C. bombycina* has the lowest LT50, significantly lower than *C. niger*, *C. mauritanica* and *C. piliscapa* which are characterized by the lowest CT_{max}; yet, the silver ant occurs in the hottest environment. In addition, and unlike the three species above, *C. bombycina* cannot heat harden to improve heat tolerance (see below).

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

Comparing heat tolerance in ectotherms is challenging because upper thermal limits are generally constrained (Hoffmann et al., 2013) and tolerance (CT_{max}) values were often characterized using different methods (Roeder et al., 2021). In ants, some studies have examined thermal tolerance with the same method as the one used here. In the tropical ant genus *Atta*, workers' survival rate reaches 40%–60% after 3 h at 37°C (Bouchebti et al., 2015). A similar survival rate was obtained at 43°C in urban populations of *A. sexdens* (Angilletta et al., 2007). In the Australian ant *Iridomyrmex purpureus*, workers reach a survival rate of 25% after 2 h at 45.8°C (Andrew et al., 2013). All these species display a lower heat tolerance compared with *Cataglyphis*. Similar thermal tolerance to *Cataglyphis* has been reported in *Monomorium pharaonis*, *M. floricola* and *Tetramorium bicarinatum* (Solis & Bueno, 2012). In the desert ants *Pogonomyrmex rugosus* and *P. barbatus*, 50% of alate females reportedly survive for 2 h at 47°C (Johnson 2000).

We found that heat hardening increased heat tolerance in the three species (*C. piliscapa*, *C. niger* and *C. mauritanica*) occurring in habitats with lower maximum annual temperatures (MaxT). In contrast, the three species that suffered from heat loading (*C. bombycina*, *C. holgerseni* and *C. cubica*) are those that endure higher maximum annual temperatures. This result suggests a trade-off might exist between the ability to tolerate extremely high temperatures and the ability to enhance thermotolerance by means of heat hardening. These findings fit with similar observations on other ectotherms showing a trade-off between high thermal tolerance and hardening capacity (reviewed in van Heerwaarden and Kellerman 2020). They are also consistent with field observations indicating that species able to more readily exploit heat hardening have longer daily foraging windows (*C. piliscapa*, Cerdá et al., 1989; *C. mauritanica*, Knaden & Wehner, 2005; *C. niger*, S.A. pers. obs.) compared with species that can less readily exploit heat hardening (*C. bombycina*, Wehner et al., 1992; *C. holgerseni* and *C. cubica*, R.P. pers. obs.).

We found that the HSR in *Cataglyphis* involves a wide range of cellular pathways and protective mechanisms. It appears that heat tolerance may be influenced by the regulatory dynamics of four major co-expressed biological pathways: (i) the regulation of transcriptional machinery and DNA metabolism, (ii) proteome safeguarding and restoration, (iii) the digestion and elimination of toxic residues and (iv) the preservation of cellular integrity. Each pathway is addressed below.

(i) Many of the genes found to be associated with the HSRs in *Cataglyphis* are involved in transcription (e.g., *nuclear protein 1*, *interferon regulatory factor 2 binding protein 2-like* and *dna-directed rna polymerase*); epigenome regulation (e.g., *histone h2a*, *histone h3*, *cysteine-rich protein 2-binding protein*, *methyltransferase-like protein*, *nad-dependent deacetylase sirtuin 1* and *something about silencing protein 10*); and RNA or DNA metabolism (e.g., *rna-dependant helicase*). Some of these genes are also involved in DNA preservation and repair. For example, histone H2A and histone H3 are known to help safeguard and fix damaged DNA (Bungard et al., 2010; Delaney et al., 2018; Foster & Downs, 2005). In addition, DNA repair and preservation may be influenced via the general regulation of *dna repair protein rad51 homolog*, *dna repair protein xrcc4-like* and *dna mismatch repair protein msh6* (Shinohara et al. 1992; Marsischky et al., 1996; Yurchenko et al., 2006); in *C. cubica*, *C. piliscapa* and *C. niger* such functions might also be fulfilled through differential regulation of *nad-dependent deacetylase sirtuin 1* (Mei et al., 2016). Overall, the broad diversity of genes associated with the regulation of transcription and DNA metabolism suggests that the HSRs are rapid and transient, providing dynamic and efficient molecular protection.

(ii) We observed the differential expression of genes in all the *hsp* families (except Hsp100). This result suggests that safeguarding and restoring the proteome is crucial during heat stress. The three types of heat-shock proteins—Hsp70s, Hsp90s and sHSPs—were all commonly expressed in *Cataglyphis* (except in *C. holgerseni*). Among the sHSPs, protein lethal(2)essential for life prevents the aggregation of misfolded proteins and ensures the protection of the actin cytoskeleton (Mounier & Arrigo, 2002; Willot et al., 2018). Hsp70s and Hsp90s actively participate in the protein-folding process; Hsp90s also stabilize the proteasome system (Evgen'ev et al. 2014). In ants, social bees and social wasps, *hsp83* (part of the Hsp90 family) and *hsc70-4* (part of the Hsp70 family) have a unique evolutionary history—changes in *cis*-regulatory elements have given rise to various inducible forms (Nguyen et al., 2016). Consequently, it may be that highly inducible forms of *hsp90* and *hsp70* were selected for in *Cataglyphis* in response to hot habitat conditions. Notably, the upregulation of the transcription factor *interferon regulatory factor 2* decreases interferon levels, resulting in an increase in Hsp70 levels (Kubo et al., 1996). This finding points to the fundamental role of the Hsp70 family in HSRs. Other chaperones, such as *dnaj* (Hsp40 homologue), *PPIA* (peptidylprolyl *cis-trans* isomerase) and *bag family molecular chaperone* (Hsp70 cochaperone) were significantly coregulated in all six species, indicating the presence of diverse mechanisms for safeguarding the proteome.

(iii) Part of proteostasis is the digestion and elimination of toxic residues, such as protein aggregates and ROS. Here, we found that heat stress induces the upregulation of genes involved in the ubiquitin proteasome system (UPS), such as *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 et al. 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 such as 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 (Ma et al., 2019; Pandian et al., 2020; Xing et al., 2013).

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

The regulation of these four biological pathways in response to heat stress is not unique to the ant genus *Cataglyphis*. Indeed, histone metabolism modifications inducing chromatin rearrangement and regulating transcriptional gene expression have been reported

in yeast, plants and animals (Delaney et al., 2018; Kim et al., 2015; Viéitez et al. 2020). Likewise, upregulation of transcripts involved in the protein folding machinery, antioxidative defence and proteasome activity has been documented from bacteria to mammals (Evgen'ev et al. 2014). For example, in the copepod *Tigriopus californicus*, Schoville et al. (2012) reported upregulation of Hsp and proteins involved in proteolysis. Similar results were documented in the hydrothermal vent decapod *Shinkaia crosnieri*, in which thermal stress upregulates transcripts encoding Hsp, proteins involved in proteolysis, antioxidants and immune defences (Cheng et al., 2019). In the ant *Aphaenogaster carolinensis*, Stanton-Geddes et al. (2016) showed that heat stress induces a molecular response involved in apoptosis and cellular reorganization (Stanton-Geddes et al., 2016). However, these authors did not distinguish the specific genes and molecular mechanisms involved in the response. Recently, particular attention was also devoted to upregulation of Hsp, antioxidants and proteinases under heat stress in nonheat-adapted organisms, such as the honeybee (Ma et al., 2019), the ant *Prenolepis imparis* (Tonione et al., 2020) or the moth *Ostrinia furnacalis* (Chen et al., 2019). While regulation of the transcriptional machinery/DNA metabolism, the proteome safeguarding/restoration and the digestion/elimination of toxic residues have been frequently reported in response to heat stress, membrane modifications and cytoskeletal rearrangements seem much less common (Cui et al., 2019; Lü & Huo, 2018; Zhang et al., 2015; Zheng et al., 2019). Upregulation of such genes was recently reported in canola *Brassica napus* (Gao et al., 2021), the leafminer *Liriomyza trifolii* (Chang et al., 2020) and the black sea cucumber *Holothuria leucospilota* (Li et al., 2021).

In conclusion, we discovered high thermal tolerance and similar HSR across *Cataglyphis* ants from different phylogenetic groups, despite the differences in the thermal regimes they experience in nature. We found that a range of biological processes—including the regulation of genes involved in transcription and DNA metabolism, proteome stability, the elimination of toxic residues, and the maintenance of cellular integrity via membrane modifications and cytoskeletal rearrangements—are conserved in *Cataglyphis* HSR. Although we did not explicitly test the functional role of the transcriptomic response in heat adaptation, we hypothesize that the suite of molecular HSR may mechanistically contribute to the high thermal tolerance of *Cataglyphis* species, allowing these ants to forage even when temperatures outside are close to their physiological limits. Further research, using similar methods, in other highly thermophilic and nonheat-tolerant ant species should examine whether such cellular and molecular adaptations are somewhat conserved or convergent when compared to other taxa.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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. analysed 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.

DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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