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Facing lethal temperatures: heat shock response in desert and temperate ants

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1. Abstract

Several genera of desert ants have adapted to endure prolonged exposure to high temperatures. The study of these ants is thus essential to unravel how species respond and adapt to thermal stress. We investigated the thermal tolerance and the transcriptomic heat stress response of three desert ant genera adapted to withstand high temperatures (*Cataglyphis*, *Melophorus* and *Ocymyrmex*) and two temperate genera (*Formica* and *Myrmica*) to explore convergent and specific adaptations. We found a variable transcriptomic response among desert species exposed to similar levels of physiological heat-stress: *Cataglyphis holgerseni* and *Melophorus bagoti* differentially regulated very few transcripts (54 and 53, respectively), while *Cataglyphis bombycina* and *Ocymyrmex robustior* showed greater expression alterations (affecting 253 and 698 transcripts, respectively). These two responsive mechanisms of lower and higher gene expression changes were related to desert species thermal tolerance survival pattern and convergently evolved in distinct desert ant genera. Moreover, the two temperate species tested differentially expressed thousands of genes more than desert ants in response to heat stress, suggesting that keeping restrained gene expression is an important adaptation in heat adapted species. Finally, we found a significant overlap of the molecular pathways activated in response to heat-stress in temperate and desert species. This indicates that the molecular processes involved in heat-stress survival appears to be evolutionary conserved in ants, even though taxonomically restricted genes also participated in the heat stress response.

2. Introduction

Global climate changes affect species distribution, adaptation, and survival (Sala et al., 2000). Ectotherm species in particular are highly sensitivity to temperature increase, and even moderate warmer temperatures may lead to a disproportionate increased heat failure (Jørgensen et al., 2022). To establish and improve predictive models and management plans to manage biodiversity loss, it is therefore primordial to understand the effects of thermal stress in different organisms (Chevin et al., 2010, Jørgensen et al., 2022). A major effect of severe heat stress is the denaturation of the three-dimensional structure of macromolecules such as proteins and cell membranes, leading to function loss, metabolic failure and, ultimately, cellular and organism death (Lepock, 1997; Kokura et al., 2016). To face heat-stress, the cellular machinery may activate multiple pathways increasing the production of proteins to limit or repair cell damage (Richter et al., 2010). The best-known examples of proteins involved in this response are the heat-shock proteins (HSP). These proteins perform several functions essential to survival under heat stress; they ensure correct refolding of denaturated proteins, prevent harmful protein aggregation (due to the association of misfolded proteins), and participate in the elimination of protein aggregates (Evgen'ev et al., 2014). Other pathways triggered by high temperature include that of unsaturated longer fatty acids and sterols accumulation, whose increased production helps to keep the fluidity of the cell matrix and of the membrane (Hazel, 1995). Likewise, the production of antioxidants and detoxification enzymes, like super oxide dismutase (SOD) or glutathione peroxidase (GPx), help reducing the accumulation of reactive oxygen species (ROS) (Birben et al., 2012). Under heat stress, these molecular pathways interact composing an intricate multi-level response that, despite its complexity, must be rapidly activated to optimize survival (Perez et al., 2021). This response is expected to be particularly important in small ectotherms – like insects – whose body temperature is highly correlated to that of their local environment (Angilletta, 2009).

Hot deserts are among the most stressful habitats on Earth, organisms inhabiting these regions must cope with extremely high temperatures and low humidity (Laity, 2009). These conditions have led most desert animals to adopt nocturnal or bimodal activity patterns, exploiting the cooler dawn and twilight hours to avoid the scorching heat (Marsh, 1988; Costa, 2012). Amazingly, several ant species display the exact opposite behaviour; they only leave the nest during the warmest hours of the day when air and ground temperature are close to their own lethal thermal limits (Cerdá et al., 1998; Wehner et al., 1992; Wehner, 2020). Such unusual activity pattern allows these insects to forage for heat-struck arthropods while reducing competition and predation pressures from other desert organisms. This so called "thermal scavenging" behaviour has appeared at least three distinct times in phylogeographically distant heat adapted ant genera: (1) within the *Cataglyphis* in the deserts of the northern hemisphere, (2) in some *Melophorus* species from the Australian outback, and (3) among the *Ocymyrmex*

ants who inhabit deserts of sub-tropical Africa (Figure 1; Marsh, 1985; Andersen, 2007; Boulay et al., 2017). Workers of those genera exhibit similar behavioural and morphological adaptations likely resulting from a convergent evolutionary process in response to their peculiar thermal niche (Wehner & Wehner, 2011). They can withstand body temperature exceeding 45°C during a long period of time (Christian & Morton, 1992; Turner et al., 2000; Willot et al., 2017); their legs are considerably longer relatively to their body size, which maximizes the distance between their body and the burning ground and allows higher running speed reducing foraging time and enhancing forced convective cooling (Sommer & Wehner, 2012; Centorame et al., 2019); and they periodically exploit thermal refuges in shaded areas or ground elevated places to benefit from cooler spots (Marsh, 1985). Recently, our group has characterized the transcriptomic heat shock response of six *Cataglyphis* species from different habitats and thermal regimes (Willot et al., 2018; Perez et al., 2021). We found that the *Cataglyphis* heat stress response builds upon the co-expression of gene clusters mainly involved in proteome stability, elimination of toxic residues, DNA/RNA metabolism and the maintenance of cell integrity through membrane modification and cytoskeletal rearrangements. Whether such molecular adaptations/pathways to cope with heat stress are genera-specific or convergently evolved in different desert ants remains however unknown.

Herein, we investigated the thermal tolerance and the transcriptomic response to heat stress of four species of desert ants belonging to the genera *Cataglyphis*, *Melophorus* and *Ocymyrmex* (Figure 1). Additionally, to explore convergent and specific adaptations to heat-stress, we compared the heat-shock response found in desert ants with that of related outgroup temperate species from the Formicinae (same subfamily as *Cataglyphis* and *Melophorus*) and Myrmicinae (same subfamily as *Ocymyrmex*). For each species, we first measured their heat tolerance by assessing the survival rate of workers at increasing temperatures and established the species thermal limit. Then, we identified genes involved in the heat-shock response by quantifying the differentially regulated transcripts between heat stressed and control workers. Lastly, we performed cross-species comparisons using differential gene expression and survival results to identify whether any convergence could be observed in the heat shock response of desert species. Our results show that desert ants benefit from two main molecular strategies to deal with high temperatures. First, a constitutive high expression level of heat stress genes; this response is associated with individuals' survival decreasing rapidly after reaching their thermal limit. Second, a reactive gene expression regulation in response to increasing temperatures that was associated with a wider variation in individual survival capacity above species thermal limit. Moreover, unlike temperate species, desert ants maintain strong gene expression control even at considerably higher temperatures, indicating this is a key adaptation to withstand heat stress.

3. Results

3.1. Desert ants show two survival patterns aligned to two distinct gene expression regulation responses under heat stress.

During heat stress assays, the median lethal temperature after 3h exposure (LT50_3h) differed significantly among species (Figure S1). It was 39.91°C (CI 95%: 0.16) for *Myr. sabuleti*; 42°C (0.17) for *F. fusca*; 44.83°C (0.18) for *C. bombycina*; 46°C (0.16) for *C. holgerseni*; 46.8°C (0.16) for *O. robustior*; and 49.83°C (0.15) for *Mel. bagoti*. Consistently, the temperature below which the survival rate was significantly different from 100% after 3h exposure (upper thermal limit, UTL_3h) was also the lowest for the two temperate species, *Myr. sabuleti* and *F. fusca*, at 39°C and 41°C respectively. *Mel. bagoti* was the most heat tolerant ant species with 95% of workers surviving at 49°C for 3 hours. The other ants showed intermediated UTL_3h values; *C. holgerseni* had a UTL_3h of 45°C, and both *C. bombycina* and *O. robustior* an UTL_3h of 43°C. For all species, the survival ratio markedly dropped from about 100% to 0% within a two-degree temperature increase interval (Figure 2), except for the Sahara ant *C. bombycina* and the Namib ant *O. robustior*, whose survival gradually decreased within a wider temperature range of six degrees.

Reference transcriptome sizes varied from 38,726 in *Mel. bagoti* to 84,784 in *Myr. sabuleti* (Figure 3B, major quality parameters are given in Table S2). The number of transcripts differentially expressed (DET) between the HS and NHS conditions ranged from 53 in *Mel. bagoti* to 10,778 in *Myr. sabuleti* (Figure 3A; complete lists of DET in each species and their annotation are provided in Supplementary Files 2–13). Based on the range of differential gene expression observed across the species, we grouped their responses into three responsive profiles that were not phylogenetically conserved (Figure 4): the first comprised by *Mel. bagoti* and *C. holgerseni* both showing weak heat stress expression responses, with only 53 and 54 DET respectively; the second included *C. bombycina* and *O. robustior* which had a medium heat stress expression response of 253 and 698 DET, respectively; and the third contained *F. fusca* and *Myr. sabuleti* which had strong heat stress expression response and differentially expressed 4,991 and 10,778 transcripts, respectively. Despite the correlation between transcriptome size and the number of transcripts differentially expressed in response to heat stress (*Pearson R*= 0.99, p = 6e−05, Figure S2), temperate species showed a minimum seven-fold increase in gene expression activity during assays in comparison to desert ants (Figure 3A), while the transcriptome size is only one-fold greater (Figure 3B). Moreover, if we consider only the desert species, the correlation between transcriptome size and the number of DET is lost (*Pearson R*= 0.63, p = 0.37, Figure S2).

Thus, desert ants displayed a more restrained gene expression regulation in response to thermal stress than temperate species, and transcriptome size alone cannot explain such discrepancy. Furthermore, wider gene expression alterations in response to heat stress were

linked to larger variation in workers' survival: desert ants with a more intense expression response (*i.e.*, *C. bombycina* and *O. robustior*; Figure 4) displayed a more gradual decrease in survival ratio (Figure 2) – dropping from 100% to 0% survival within a six-degree temperature interval – while species with weak heat stress expression response (*i.e.*, *C. holgerseni* and *Mel. bagoti*) showed a marked reduction in survival within a smaller temperature range of two degrees. In temperate species, survival also decreased rapidly within a two-degree temperature increase; however, in contrast to desert ants, many genes where differentially expressed.

3.2. Fewer gene expression alterations in response to heat stems from high constitutive levels of heat stress related genes in desert ants.

To further investigate whether the different expression patterns found among desert ants were due to a lack of expression of heat stress related genes, or instead, to a distinct timing regulation of these genes, we focussed on the expression pattern of admittedly known heat stress related genes – the heat shock proteins 70 and 90 (HSP70 and HSP90) – and inspected the expression of their related transcripts. The expression pattern plots of these transcripts in all species are reported in supplementary files S14 to S19. Although expression counts of desert ants HSP70 and HSP90 transcripts under heat stress and non-heat stress conditions were only significantly differentially regulated in *C. bombycina* and *O. robustior*, they were still expressed at a high level in *C. holgerseni* and *Mel. bagoti* (Figure 5). In these two latter species, however, expression levels of HSP70 and HSP90 transcripts in the control group (non-heat stressed ants) were already elevated, which explains the lack of significant differences in their expression response. These results indicate that desert species with fewer gene expression alterations (*i.e.*, *C. holgerseni* and *Mel. bagoti*) have a constitutive up regulation of HSP70 and HSP90 and do not significantly escalate their expression levels when exposed to increasing temperatures, unlike *C. bombycina* and *O. robustior*.

These findings suggest that the reduced number of differentially expressed genes under heat stress in *C. holgerseni* and *Mel. bagoti* stems from a constitutive upregulation of heat stress related genes. Thus, the distinct expression patterns observed in desert ants would reflect a difference in timing regulation of heat stress related genes with *C. holgerseni* and *Mel. bagoti* constantly keeping a higher expression of these genes, while *C. bombycina* and *O. robustior* upregulate them in response to heat stress.

3.3. Molecular pathways responsive to heat stress in desert ants with greater gene expression changes are also activated in temperate species.

Among desert ants, only *C. bombycina* and *O. robustior* had a significant number of DET in common (Figure 3C). When including the ant species from temperate regions in the

193 comparisons, there was a significant number of DET in common among all species except *C.*
194 *holgerseni* and *Mel. bagoti* (Table S3). Species pair comparisons containing all genes found
195 to be differentially expressed in common across species follows in supplementary file S20.
196 Similarly, from the GO analyses (Table S4, Figure S3) we found that terms associated with
197 protein folding ("Protein folding", "Protein refolding" and "*de novo* protein folding", respectively
198 GO:0006457, GO:0042026 and GO:0006458) and cellular response to heat (GO:0034605)
199 were commonly enriched in *O. robustior*, *Myr. sabuleti* and *F. fusca*. In *C. bombycina,* although
200 several *hsp* genes were upregulated (supplementary file S2), protein folding GO terms were
201 not significantly enriched using our adjusted *p*-value cut-off at < 0.01 ("Protein refolding", *p*-
202 value= 0.025). Between *Myr. sabuleti* and *F. fusca*, the two species with a much greater
203 number of enriched GO terms (Table S4, Figure S3), biological processes involved in
204 mitochondria homeostasis, actin or microtubules organisation, lipid metabolism, cellular
205 signalling and transduction, and DNA/RNA metabolism overlapped. When we categorized the
206 differentially expressed genes into the most frequent functional categories (Figure S4), instead
207 of only focusing on significantly enriched GO terms, we see that for all species most of the
208 characterized differentially expressed genes are commonly comprised within the same
209 categories being the larger proportion of them involved with cytoskeletal rearrangement and
210 DNA/RNA metabolism. Notably, *C. holgerseni* and *Mel. bagoti* did not differentially regulated
211 any gene involved in chaperoning and autophagy. Thus, for the 4 species with a wider gene
212 regulation response (*i.e.,* the desert species *C. bombycina* and *O. robustior* and the temperate
213 species *Myr. sabuleti* and *F. fusca*), there was a conserved differential regulation involving
214 similar genes and pathways in response to heat stress.

215 To better visualise the metabolic and biochemical pathways of heat-shock response, we
216 analysed the KEGG pathways of DET (FCx2) for each species and checked the biological
217 function of each KEGG ortholog (KO) assigned. Overall, *C. bombycina* (7 KO), *C. holgerseni*
218 (4 KO) and *Mel. bagoti* (5 KO) displayed few KEGG pathway matches. *C. bombycina* and *C.*
219 *holgerseni* exhibited an upregulation of glutathione synthesis through its precursor [for *C.*
220 *bombycina*: glycine and cysteine/methionine metabolism; for *C. holgerseni*: cysteine-glycine
221 synthesis]. In addition, *C. holgerseni* showed a match for C10-20 isoprenoids biosynthesis
222 (M00367). In contrast, 51 KO were assigned for *O. robustior* including genes involved in
223 glutathione synthesis or its precursors (K00799, K00830 or K00273), lipid metabolism [Inositol
224 phosphate metabolism (M00130, M00131, M00132), fatty acid synthesis and elongation
225 (M00082 and M00083), and triacyl glycerol metabolism (M00089)], sugar metabolism [glucose
226 metabolism (K01189 and K00008), trehalose synthesis (K16055 and K00963), pentose
227 phosphate pathway (K00115 and K01623)], proline synthesis (K0086) and lysine degradation
228 (K01435). For *F. fusca* and *M. sabuleti,* we found 88 KO and 148 KO, respectively. For both
229 species, KO included genes involved in sugar metabolism [glycolysis (M00001) and citrate

230 cycle (M00010)], pentose phosphate pathway (M00165 and M00004), NADP/NADPH
231 recycling (M00172), fatty acid elongation (M00083 and M00085), tryacyl glycerol and ceramide
232 synthesis (M00089 and M00094 respectively and methionine salvage pathway (M00034). In
233 addition, *F. fusca* showed an enrichment in acyl glycerol and leucine degradation (M00098 and
234 M00036) and *Myr. sabuleti* in inositol phosphate metabolism and ketone synthesis (M00130
235 and M00088).
236
237 **3.4. Greater gene expression changes are associated with increased participation of**
238 **taxonomically restricted genes in the heat stress response.**
239 As documented above, cross-species comparisons of gene annotation and functional
240 categories showed that conserved genes and pathways are largely involved in the heat stress
241 response. Nevertheless, our results also revealed several uncharacterized genes that were
242 differentially regulated suggesting that new genes may also play a role in heat adaptation
243 (Figure S4). To further investigate this issue, we accessed the transcripts orthology based on
244 their protein sequence (using OrthoFinder) and considered lineage specific orthogroups as
245 taxonomically restricted, hence, new genes. From the 292,232 protein coding genes analysed
246 across all 6 species studied, 266,366 (91.1%) were assigned to at least one of the 34,135
247 orthogroups identified. Species-specific orthogroups accounted for 9,178 (26.9%) of all
248 orthogroups and contained 29,645 genes (10.1% of the total), while 8,753 orthogroups
249 contained all species and only 321 were single copy (Table S5). Compared to genomic
250 orthology estimations (Darras et al., 2022), the transcriptomic based orthology analyses
251 resulted in more species-specific and less single copy orthogroups which can be expected
252 since transcriptomic data include multiple isoforms per gene (see BUSCO duplication ratio at
253 Table S2). For this reason, we estimated the relevance of new genes not only based on their
254 frequency but comparatively, by contrasting the relative proportion of transcripts in each
255 orthogroup category between the transcriptome and among the DET. We expected that any
256 bias in orthology estimation due to the transcriptomic nature of the data affects the proportion
257 in the transcriptome and its subsets in a similar manner. Thus, our null hypothesis was that the
258 proportion of transcripts in each orthology category would be equivalent in the entire
259 transcriptome and among the differentially expressed transcripts.
260 According to the OrthoFinder resulting table (supplementary file S21), we filtered
261 orthogroups from five categories: I- orthogroups shared by all ants, II- orthogroups shared only
262 by Myrmicinae ants, III- orthogroups shared only by Formicinae ants, IV- orthogroups shared
263 only by the two *Cataglyphis* species, and V- species-specific orthogroups. Categories I to III
264 included 8,753, 1,055 and 324 orthogroups respectively, and were considered as
265 representative of taxonomically conserved genes. While categories IV and V represented
266 taxonomically restricted genes. Category IV included 890 orthogroups. In category V, the

267 number of species-specific orthogroups found for each species varied from 712 in *C.*
268 *bombycina* to 3,053 in *Myr. sabuleti* (all species values are given Table S5). In species with
269 medium and high gene regulation responses, namely *C. bombycina*, *O. robustior*, *Myr. sabuleti*
270 and *F. fusca*, we found an overall increase in the proportion of taxonomically restricted genes
271 among the DET (Figure 6). This increase was significant in *F. fusca* (Fisher's Exact Test *p*-
272 value < 2.2e-16), *Myr. sabuleti* (*p*-value < 2.2e-16) and *O. robustior* (*p*-value < 4.5e-06), but
273 not in *C. bombycina*, neither in *Cataglyphis* specific nor species-specific orthologs (*p*-values =
274 0.732 and 0.086, respectively). Contrastingly, a reduction of taxonomically restricted genes
275 among the differentially expressed transcripts was observed in *C. holgerseni*, and in *Mel.*
276 *Bagoti* only a slight increase of less than 1% in the ratio of new genes among the differentially
277 regulated genes was found (Figure 6).

278 These findings suggest that the involvement of taxonomically restricted genes in ants heat
279 stress response is associated with the number of genes under differential expression, with
280 species that differentially regulate more genes as temperature increases also altering the
281 expression of a higher proportion of taxonomically restricted genes.

4. Discussion

We characterized and compared the heat tolerance and the heat stress transcriptomic response of four ecological equivalents desert ant species from three distinct genera, and of two outgroup species living in a temperate region. From all 6 studied species, the red honey ant *Melophorus bagoti* was the most heat tolerant, withstanding the highest temperatures ever reported in ants (Johnson, 2008; Solis & Bueno, 2012; Andrew et al., 2013; Perez et al., 2021). However, in natural habitats, the Saharan *Cataglyphis bombycina* is known to endure the most environmental heat, with a mean temperature in the warmest quarter of 30.8^{o}C and maximum temperature of 42.3^{o}C (Table S1). An important part of *C. bombycina* heat resistance mechanism in their natural habitats relies on prism-shaped hairs covering the body of workers. These hairs reflect solar radiation through total internal reflection, which limits heat absorption when the workers are exposed to sunlight (Shi et al., 2015; Willot et al., 2016). This adaptive mechanism deflects the energy associated with visible wavelengths but played no role in laboratory conditions when we measured heat-tolerance through infrared warming (see also Perez et al. 2021). Not surprisingly, the least heat tolerant species studied were those from temperate areas, namely *Myrmica sabuleti* and the dusky ant *Formica fusca*. Both species displayed the lowest upper thermal limit and LT50_3h values (respectively, UTL_3h: 39°C and 41°C, LT50_3h: 39.9°C and 42°C). In ants and other insects, there is a clear association among conditions at their microclimatic level, operative temperatures experienced by individuals, and upper-thermal limits (Sunday et al., 2014, Woods et al., 2016, Clustrella-Trullas et al., 2021). For example, species occupying niches exposed to greater variability in environmental temperatures, such as open areas and canopies, often show increased heat tolerance (Kaspari & al. 2014, Baudier & al. 2018, Bujan & al. 2020). Our results show this association seems to also occur in strict desert thermal scavenging ants, species whose peculiar thermal niche has likely driven to the evolution of higher upper thermal limits. These observations highlight the high vulnerability of ectotherms, including social insects, to temperatures changes (reviewed in Perez et al., 2020; Martinet et al., 2021, Jørgensen et al., 2022).

Temperate species differentially regulated thousands of genes more than thermophilic ones, showing that focussed gene expression under heat stress is an important adaptation of desert ants. A similar adaptative response has also been documented at the populational level; studies comparing same species populations from colder and hotter environments found that heat adapted populations show a reduced gene expression response under heat stress. Japanese mantis shrimp populations from temperate regions differentially regulate almost a thousand more transcripts in response to heat stress than shrimp populations inhabiting a warmer environment (Lou, Han & Gao, 2019). Similarly, populations of the Chinese minnow fish from cooler regions in the north show more temperature responding genes under heat

319 stress than southern populations (Yu et al., 2018). In the oilseed rape (*Brassica napus*), the
320 heat-sensitive genotype showed larger variation in the methylation pattern of multiple genes
321 under heat stress in comparison to the heat-tolerant genotype (Gao et al., 2014). Since
322 molecular damage induce the heat shock response (Evgen'ev et al., 2014), reduced gene
323 expression in heat adapted organisms might simply reflect a limited molecular damage at
324 higher temperatures. Selection is expected to favour proteins optimized to certain thermal
325 environments (Fields, 2001; Sælensminde, Halskau & Jonassen, 2009; Fields et al., 2015),
326 thus the proteome of thermophile desert species might keep protein function even at elevated
327 temperatures delaying the molecular heat shock response. A recent study on *Mytilus* mussels
328 indeed showed that small differences in habitats were sufficient to cause such protein structural
329 alterations (Chao et al., 2020). Additionally, we found in desert ants many differentially
330 expressed genes in response to heat stress that were related to DNA/ RNA metabolism and
331 regulation (Figure S4). This is an indication that protective mechanisms against uncontrolled
332 gene expression, such as DNA heterochromatinization and histone aggregation, are also in
333 place. Altogether, these results suggest that the reduced gene expression alterations observed
334 in desert ants in comparison to temperate species likely stem from a combination of diminished
335 molecular damage and increased gene expression control mechanisms at higher
336 temperatures, rather than from the activation of a single gene or pathway that evolved
337 exclusively in desert ants. In line with this, the transcriptomic response of *F. fusca* and *Myr.*
338 *sabuleti* involved many of the same cellular pathways and protective mechanisms found
339 differentially regulated in desert species. Thus, temperate species have most of the molecular
340 toolkit necessary for the heat stress response of desert ants. However, unlike the latter,
341 temperate species co-activate many other genes and pathways upon heat stress likely leading
342 to an unspecific and energetically costly response.

343 Our results suggest that desert ants evolved two adaptive strategies at the transcriptomic
344 level: (1) the differential regulation of very few genes in response to heat stress, as shown by
345 *C. holgerseni* and *Mel. bagoti*, or (2) the differential regulation of several genes upon increased
346 temperature, as observed in *C. bombycina* and *O. robustior*. In our analyses, the desert
347 species that showed the stronger expression response under heat stress also displayed larger
348 variation in temperature survival, with their survival curve gradually decreasing from 100 to 0%
349 within a six-degree interval (Figure 2). Contrastingly, survival of species with fewer gene
350 expression differences between heat stressed and non-heat stressed conditions decreased
351 more abruptly reaching 0% within a two-degree variation interval. These results suggest that
352 species with larger expression differences may have a reactive response to heat, while species
353 with fewer changes in gene expression may keep a constitutive response to heat stress.
354 Consistent with a constitutive synthesis of thermoprotective molecules, HSP70 and HSP90
355 transcripts are indeed highly expressed in non-heat stressed as well as in heat stressed

samples of *C. holgerseni* and *Mel. bagoti* (Figure 5). This strategy would provide high heat resistance, but low flexibility at their thermal limit capacity since gene expression would not be as responsive to environmental temperature changes. Interestingly, the constitutive and reactive responses were not phylogenetically conserved (Figure 4), instead they evolved convergently in different desert species genera. Other *Cataglyphis* species were reported to follow a similar response to *C. bombycina* and *O. robustior*, with a maximum of 1,118 DET (mean 561 DET) and a gradual decrease in survival upon a larger temperature range (Perez et al. 2021).

Both strategies – the reactive response and the constitutive response – provide desert ants an incredible heat resistance and could have evolved from distinct evolutionary constraints. Examples of evolutionary constraints that could lead to such differences include micro habitat differences, body-size, and metabolic trade-offs. In our observations, we found that most *C. holgerseni* and *Mel. bagoti* foragers were larger, while *C. bombycina* and *O. robustior* workers were comparatively smaller (Figure S5). Large body size in desert ants could be advantageous to maintain high thermal inertia, to store more water to prevent desiccation, and to store energetic resources to face cellular stress (Bouchebti et al., 2015; Wendt & Verble-Pearson, 2016; Johnson & Stahlschmidt, 2020). It is therefore possible that for ant species with smaller body size, a reactive gene expression response would be necessary due to reduced internal storage for keeping thermoprotective molecules. It is also important to consider that these two strategies likely involve different energetic demands. The maintenance of constitutive levels of thermoprotective genes is energetically costly, however a reactive gene expression response as temperature increases would require high amounts of energy available to be used at once to activate gene expression and to recover from the possible loss of biochemical activity from protein denaturation (Nguyen et al., 2016). Therefore, energetic trade-offs and alterations in energetic pathways could represent important constraints to the evolution of these two strategies.

Among desert ants, in *C. holgerseni* and *M. bagoti* we found respectively 3 and 4 differentially expressed transcripts involved in sugar energetic pathways. Interestingly, in *O. robustior* more than 20 transcripts were upregulated in this functional category. For example, we found genes involved in glucose synthesis (*maltase 1-like*, *alpha-glucosidase* or *alpha-amylase a-like*) and transformation (*l glucose dehydrogenase fad quinone-like* or *sorbitol dehydrogenase*). In addition, KEGG pathway analyses revealed that among the DET of *O. robustior* there was a positive regulation of trehalose synthesis and of the pentose phosphate pathway (PPP). The former is known to enhance heat and desiccation tolerance in various organisms (Jain & Roy, 2009), while the oxidative phase of PPP is used to regenerate NADPH which is required by antioxidant enzymes such as the *glutathion S-transferase* and the *cytochrome P450*, both also upregulated in *O. robustior* (Hayes, Flanagan & Jowsey, 2005;

393 Pandian et al., 2020). A positive co-regulation of sugar metabolism in response to heat stress
394 was previously documented in *Cataglyphis* (Perez et al., 2021). Similarly, among the
395 temperate species, many transcripts differentially expressed are involved in sugar metabolism
396 as well as the upregulation of the PPP pathway. These results suggests that the heat-shock
397 response in ants indeed requires a lot of energy provided by the sugar metabolism.
398 Nevertheless, we found that in *O. robustior* this response is somewhat accentuated, with many
399 of the genes and regulatory pathways involved in the antioxidative machinery along with genes
400 involved in the trehalose synthesis being differentially regulated under heat stress.
401 The overlap of genes and pathways involved in the transcriptomic response to heat stress
402 of desert and temperate ants is strong evidence of taxonomically conserved genes and
403 pathways regulating this trait. Nevertheless, our analysis also shows an increased proportion
404 of new genes amongst the DET of desert and non-desert species when compared to the
405 proportion of new genes in their entire transcriptome (Figure 6). Only desert ants with fewer
406 gene expression alterations were an exception to this pattern. These observations indicate that
407 new genes are likely to be co-expressed along with taxonomically conserved genes under
408 heat-stress, possibly because they share the same promoter regions (Kaessmann, 2010).
409 Interestingly, in our data, the larger the number of genes under differential expression the
410 larger was the proportion of new genes amongst them. This co-expression offers an
411 opportunity to gene neofunctionalization during adaptation, which is one of the most important
412 origins of orphan genes (Kaessmann 2010; Werner et al. 2018). New genes are known to be
413 particularly relevant to species adaptative response (Chen, Krinsky, and Long 2013; Johnson
414 2018; Kaessmann 2010; Taylor and Raes 2004). For example, in Antarctic notothenioid fishes
415 the ice-biding antifreeze glycoprotein (AFGP), that originated from a functionally unrelated
416 gene (the *pancreatic trypsinogen-like protease2*), has been co-opted to play a crucial adaptive
417 role in preventing blood from freezing (Cheng and Chen 1999). In ants, 10% to 30% of all
418 genes in the genome are estimated to be taxonomically restricted and in Formicidae ants
419 (Wissler et al., 2013), and evidence suggest that high ratios of *de novo* gene formation exist in
420 ants (Simola et al., 2021). It is therefore possible that taxonomically restricted genes are also
421 involved in species-specific adaptations to heat stress in desert ants. Comparative genomic
422 studies and functional analyses should further explore these genes and their putative roles in
423 heat stress adaptation.
424 In conclusion, our results show that, as opposed to temperate ant species, heat-adapted ants
425 show reduced gene expression alterations even when under extreme heat stress.
426 Furthermore, two transcriptomic strategies that evolved convergently are adopted by desert
427 ants to withstand heat stress. In *C. holgerseni* and *Mel. Bagoti*, a constitutive level of
428 thermoprotective genes appears to reduce the need for great changes in gene expression
429 when temperature increases. Contrastingly, in *C. bombycina* and *O. robustior* a larger number

of genes are differentially regulated in response to increasing heat. These two responsive mechanisms are associated with the survival pattern of these ants: the species with fewer gene expression alterations have a small variability in individual survival, with all workers dying within a two-degree interval, while species with greater expression response experience a survival reduction from 100% to 0% within a larger temperature interval of six degrees. The interplay among body size, energetic metabolism, and heat tolerance in the evolution of these two strategies deserves further exploration. Finally, this study brings some methodological insights illustrating that, in some circumstances, transcriptional differences between conditions may be insufficient to understand putative adaptative mechanisms if they are not contextualized within a phylogenetic perspective. Indeed, the expression of some genes may be constitutive and, therefore, these genes will not be detected as differentially regulated even though they may be functionally relevant to species adaptation. Clearly, transcriptome studies should consider an evolutionary approach for comparative data analyses including multiple species from different phylogenetical backgrounds.

5. Material and methods

5.1. Ant sampling and rearing conditions

We studied six ant species from different locations. Four from arid and sandy deserts: *Cataglyphis bombycina* (Morocco), *C. holgerseni* (Israel), *Ocymyrmex robustior* (Namibia) and *Melophorus bagoti* (Australia); and two from a temperate region: *Formica fusca* and *Myrmica sabuleti* (Belgium). The four desert species can be considered ecologically equivalent (Perez & Aron, 2020). They inhabit sandy desert environments with mean annual temperature (AMT) above 21°C, and warmest month recorded (MaxT) ranging from 32.3 to 42.3 °C (Table S1). We included two species of desert ants from the same genus (*C. bombycina* and *C. holgerseni*) to account for intra genus variation, as well as species from three different genera of heat adapted ants (*Cataglyphis*, *Ocymyrmex* and *Melophorus*) that evolved their adaptations independently to account for inter genera variability. The two temperate ant species, *Formica fusca* and *Myrmica sabuleti*, were chosen as comparative outgroups being, respectively, representative species from the Formicinae and the Myrmicinae subfamilies. Both these species inhabit temperate areas with AMT of 10.2°C and MaxT of 22.8°C.

We excavated 4–6 colonies for each species at sampling sites and brought them back to the lab. The colonies were kept under steady conditions: mean temperature of 25°C (± 1°C) for desert species and 18°C (± 1°C) for temperate species, light-dark cycle of 12 h/12 h, and relative humidity between 30% and 40% for all ants. Ants were fed with sugar solution provided *ad libitum*, and sliced mealworms that were provided three times per week. Colonies were maintained under these conditions one month prior to the beginning of the assays.

5.2. Heat tolerance assays

Workers heat tolerance was determined using a heat stress assay as detailed in Willot et al. (2017) and Perez et al. (2021). For each species, groups of 10 randomly selected workers were placed in glass tubes (n = 6). Because body size influences heat tolerance due to differences in relative water loss (Hood & Tschinkel, 1990; Cerdá & Retana, 2000; Clémencet et al., 2010), a wet cotton ball was added in the testing tubes to reduce ant desiccation and its effect on specimens' survival during the tests. The tubes were immersed in a water bath (SW22, Julabo GmbH, Seelbach, Germany) kept at constant temperature of 39°C, 41°C, 43°C, 45°C, 47°C, 49°C or 51°C. Tubes temperatures were 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 was recorded after 3 hours; workers were identified as moribund once they lost their locomotor ability (*i.e.*, muscular paralysis; Roeder et al., 2021). For each species, we measured two indicative parameters of heat tolerance: (1) the upper thermal limit (UTL_3h), the temperature below which the survival rate is significantly different from 100% (ANOVA test

482 followed by a Tukey's test; $p < 0.05$), and (2) the median lethal temperature (LT50_3h), the
483 temperature at which survival probability equals 50%. LT50_3h was estimated using a simple
484 logistic regression of death probability in function of the temperature. The LT50_3h, and its
485 95% confidence intervals, were compared between species using the ratio test (Wheeler, Park
486 & Baily, 2006), implemented in the *ratio_test* function from the *ecotox* package (Hlina, 2019).
487

488 **5.3. RNA-Seq library preparation and sequencing**

489 For each species, we sequenced four replicates per treatment ($n = 4$). In the heat stress
490 (HS) treatment, workers were exposed to their UTL_3h for 3 hours; in the non-heat stress
491 (NHS or control) treatment, workers were maintained in the glass tubes for 3 hours at 25°C or
492 18°C, for desert and temperate species respectively. Each sample replicate contained the
493 entire body of 10 workers randomly chosen among the different colonies of a species. Ants
494 were immediately frozen after treatments and stored at -80°C until RNA extraction. RNA
495 extractions, RNA quality assessments (determined using Bioanalyzer), RNA-seq library
496 preparations and sequencing were performed by BGI Tech Solutions (Hong Kong). Total RNA
497 was extracted from the whole body using Trizol (Invitrogen, Carlsbad, CA, USA) according to
498 manufacturer's instructions (EDTA 250mM was added to *Formica fusca* extractions to avoid
499 non enzymatic RNA degradation; Valles et al., 2012). Sequencing was performed using an
500 Illumina HiSeq 4000 System. About 25 million single reads of 50 bp in length were generated
501 per sample replicate. We additionally sequenced for each species a pool of workers (HS and
502 NHS), eggs, and pupae using a HiSeq X Ten System that was used to assemble the species
503 reference transcriptomes. About 90 million paired reads of 150 bp in length were obtained for
504 each of these species' pools.
505

506 **5.4. Transcriptome assembly and differential expression**

507 Transcriptome assembly and differential expression analyses were performed
508 independently for each species. For both *Cataglyphis* species, the transcriptome and
509 differential expression data have been already published elsewhere using the same
510 methodology described hereafter (Perez et al., 2021). Quality of all sequenced reads were
511 estimated using FastQC v0.11.7 (Andrews, 2010). For the reference transcriptome assembly,
512 we used the Trinity v2.8.4 pipeline (Grabherr et al., 2011) with the sequenced dataset from the
513 pool of workers, eggs and pupae. To increase assembly efficiency, reads were first digitally
514 normalized (20x coverage) through computational coverage systematization (Brown et al.,
515 2012). Then the transcriptome was assembled using two strategies depending on the species:
516 a full *de novo* and a reference guided assembly. For the two *Cataglyphis* species and for
517 *Formica fusca* we generated both, the full *de novo* and the reference guided assemblies. The
518 reference guided approach for the *Cataglyphis* was based on the genome of *Cataglyphis*

519 *hispanica* (assembly ASM419527v1 - Darras et al., 2022) and that of *F. fusca* in the genome
520 of *Formica exsecta* (assembly ASM365146v1 - Dhaygude et al., 2019). These two
521 independent assemblies were then concatenated. For *Ocymyrmex robustior*, *Melophorus*
522 *bagoti* and *Myrmica sabuleti* only the full *de novo* assembly was performed due to lack of a
523 close reference genome. Transcripts redundancy was initially removed with CD-Hit v4.8.1
524 (Huang et al., 2010) using the threshold of 95% nucleotide similarity, then Corset v1.08
525 (Davidson & Oshlack, 2014) and Lace v1.13 (Davidson et al., 2017) were used to cluster
526 transcripts into superTranscripts, which further reduced transcript redundancy and improved
527 posterior gene expression counts (Davidson et al., 2017). Assemblies were then annotated
528 with Annocript v2.0.1 (Musacchia et al., 2015) in tandem with the UniProt Reference Clusters
529 (UniRef90) and the UniProtKB/Swiss-Prot (Suzek et al., 2015) databases (March 2019
530 versions). Only transcripts potentially encoding proteins – based on ORF estimation or blast
531 results – were retained in the final reference transcriptome assemblies. All the parameters
532 used in the assembly and annotation pipelines were the programmes' suggested defaults,
533 unless otherwise stated. Assessments of assembly quality were obtained with BUSCO v5.3.2
534 (Simão et al., 2015), against the Hymenoptera (odb10) database, and with QUAST v5.0.2
535 (Gurevich et al., 2013).
536 For the differential gene expression analyses, reads from ants of the HS and NHS
537 treatments were aligned to the reference assembly and counted using Salmon (Patro et al.,
538 2017). The transcripts that were differentially expressed between treatments were identified
539 using the edgeR (Robinson et al., 2009) and the DESeq2 (Love et al., 2014) pipelines. To
540 increase stringency and reduce the rate of false positive results, the differentially expressed
541 transcripts were selected based on the overlapping results of both analyses (*i.e.*, overlapping
542 results from edgeR and DESeq2) and using a cut-off of mean absolute $\log_2$-fold change ≥ 2
543 between treatments and FDR ≤ 1e-3 (as suggested in the Trinity pipeline for *de novo*
544 transcriptomes). We then computationally compared the list of differentially expressed
545 transcripts (DET) between each species pair based on their UniRef90 gene name annotation
546 using R (*stringr* library). Only unique, non-redundant and meaningful gene annotations (*i.e.,*
547 genes whose annotation did not contain the phrase "uncharacterised protein") were included
548 in this comparison and in the statistical tests. After this computational comparison of gene
549 names, the final list of overlapping genes was manually curated to avoid partial or redundant
550 annotation matches. Significance of common differentially expressed genes between species
551 was assessed using 10,000 random samplings comparisons of the same datasets and a cut-
552 off *p*-value < 0.01 (Rscript available at
553 https://github.com/nat2bee/Foragers_vs_Nurses/blob/master/Statistics/common.stats.R.
554
555 **5.5. Functional analyses of the differentially expressed genes**

556 To test whether gene ontology (GO) terms for biological processes (BPs) were enriched
557 among the differentially expressed transcripts, we kept only transcripts displaying non-zero
558 expression in the treatment groups (i.e., for *C. bombycina*: 29,965 transcripts; for *C. holgerseni*:
559 26,769 transcripts; for *Mel. bagoti*: 38,726 transcripts; for *O. robustior*: 45,701 transcripts; for
560 *M. sabuleti*: 84,784 transcripts; and for *F. fusca*: 62,416 transcripts). We used the "weight01"
561 algorithm with the Fisher enrichment test in the R package TopGO (Alexa & Rahnenfuhrer,
562 2016) for the enrichment test, and significance was evaluated based on an adjusted alpha
563 level of 0.01. For summary visualization of enriched GO terms in desert and non-desert
564 species, we used the Revigo server and the *Drosophila* database (Supek et al., 2011).
565 For each species, the function of all differentially expressed transcripts was manually
566 checked and categorized according to their metabolic and cellular role from which we selected
567 nine most frequent categories: Apoptose, Authophagy, Chaperoning, Cytoskeletal
568 rearrangement, DNA/RNA metabolism, Lipid metabolism, Proteolysis, ROS elimination, Sugar
569 metabolism, Other and Uncharacterized (*i.e.,* genes of unknown function or not annotated).
570 Finally, we assessed gene involvement in specific biochemical and metabolic pathways using
571 the Kyoto Encyclopedia of Genes and Genomes (KEGG), via the KEGG Automatic Annotation
572 Server (KAAS; Moriya et al., 2007).
573
574 **5.6. Expression levels of Heat shock proteins**
575 We filtered transcripts in the reference transcriptome annotated to the heat shock protein
576 genes HSP70 and HSP90 using a custom script (Find_Genes.py available at
577 https://github.com/nat2bee/Cataglyphis_HS/tree/main/Scripts) based on representative
578 search terms annotation (*i.e., Hsp70*, *Hsp 70*, *Heat shock protein 70*, *Hsp90*, *Hsp 90*, *Heat*
579 *shock protein 90*). For each of these transcripts, we then plotted their normalized expression
580 count in TMM from all heat stressed and non-heat stressed samples for visualization.
581
582 **5.7. Orthologous and new genes identification**
583 We used TransDecoder v5.5.0 (Haas et al., 2013) to convert transcriptome ORF regions
584 to amino acid sequences. Using OrthoFinder v2.5.4 (Emms & Kelly, 2019) with default
585 parameters, we then estimated orthology among the transcriptomes of all species and
586 generated the species phylogenetic tree using MAFFT for sequence alignment and FasTree
587 for tree estimation. Orthogroups containing at least one transcript differentially expressed were
588 considered as a differentially expressed orthogroup. We classified the orthogroups into five
589 categories filtering the OrthoFinder resulting table: I- orthogroups containing representative
590 transcripts from all ants, II- orthogroups containing representative transcripts only in
591 Myrmicinae ants, III- orthogroups containing representative transcripts only in Formicinae ants,
592 IV- orthogroups containing representative transcripts only in the two *Cataglyphis* species,

accounting for intra genus similarities and V- species-specific orthogroups. Transcripts within orthogroups in categories IV and V were considered as taxonomically restricted genes, while categories I, II and III were composed by conserved orthologous. For each species, only the relevant categories were considered. Fisher's Exact Test was used to test whether the proportion of genes in each of these categories diverged significantly between the differentially expressed transcripts and the entire species transcriptome.

5.8. Data availability

Sequenced data and reference transcriptome assemblies for the studied ant species are available at NCBI under the Bioproject PRJNA632584; the annotations for these assemblies and scripts used are accessible at GitHub (https://github.com/nat2bee/Cataglyphis_HS).

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Conflict of interest

The authors declare no conflict of interest.

Author Contributions

RP, SA and WQ conceived the study and collected samples. NSA and MD designed the bioinformatic analysis which were performed by NSA. RP and NSA analysed the data. All authors contributed to drafting the article, approved the submitted version and agreed to be held accountable of all aspects of the work.

7. References

Addo-Bediako A, Chown SL, Gaston KJ. 2000. Thermal tolerance, climatic variability and latitude. *Proceedings of the Royal Society of London* 267:739–745.

Alexa A, Rahnenfuhrer J. 2016. TopGO: Enrichment analysis for gene ontology. R package.

Andersen AN. 2007. Ant diversity in arid Australia: a systematic overview. In: Snelling, R. R., Fisher, B. L. & Ward, P. S. (eds) Advances in ant (Hymenoptera: Formicidae) systematics: homage to E. O. Wilson—50 years of contributions, 80. *Memoirs of the American Entomological Institute*, Gainesville (FL). p. 19–51

Andrews S. 2010. FastQC: A Quality control tool for high throughput sequence data.

Andrew NR, Hart RA, Jung MP, Hemmings Z, Terblanche JS. 2013. Can temperate insects take the heat? A case study of the physiological and behavioural responses in a common ant, *Iridomyrmex purpureus* (Formicidae), with potential climate change. *Journal of Insect Physiology* 59:870–880.

Angilletta Jr MJ. 2009. *Thermal Adaptation. A theoretical and Empirical Synthesis*. Oxford University Press.

Baudier KM, D'Amelio CL, Malhotra R, O'Connor MP, O'Donnell S. 2018. Extreme insolation: Climatic variation shapes the evolution of thermal tolerance at multiple scales. *The American Naturalist* 192:347–359.

Birben E, Sahiner UM, Sackesen C, Erzurum S, Kalayci O. 2012. Oxidative stress and antioxidant defense. *World Allergy Organisation Journal* 5:9–19.

Bouchebti S, Jost C, Caldato N, Forti LC, Fourcassié V. 2015. Comparative study of resistance to heat in two species of leaf-cutting ants. *Insectes Sociaux* 62:97–99.

Boulay R, Aron S, Cerdá X, Doums C, Graham P, Hefetz A., Monnin T. 2017. Social life in the desert: the case study of *Cataglyphis* ants. *Annual Review of Entomology* 62: 305–321.

Brown CT, Howe A, Zhang Q, Pyrkosz AB, Brom TH. 2012. A reference-free algorithm for computational normalization of shotgun sequencing data [Genomics]. *Genome Announcements* 2:1–18.

Bujan J, Roeder KA, Beurs K, Weiser MD, Kaspari M. 2020. Thermal diversity of North American ant communities: Cold tolerance but not heat tolerance tracks ecosystem temperature. *Global Ecology and Biogeography* 29:1486–1494.

Centorame M, Angelino D, Bonanni R, Fanani A. 2019. Static and evolutionary allometry in the Italian endemic ant species *Cataglyphis italica* (Emery1906). *Ethology Ecology & Evolution* 32:16–28.

Cerdá X, Retana J, Cros S. 1998. Critical thermal limits in Mediterranean ant species: trade-off between mortality risk and foraging performance. *Functional Ecology* 12:45–55.

Cerdá X, Retana J. 2000. Alternative strategies by thermophilic ants to cope with extreme heat: individual versus colony level traits. *Oikos* 89:155–163.

663 Chao YC, Merritt M, Schaefferkoetter D, Evans TG. 2020. High-throughput quantification of
664 protein structural change reveals potential mechanisms of temperature adaptation in
665 *Mytilus* mussels. *BMC Evolutionary Biology* 20:28.
666 Chen S, Krinsky BH, Long M. 2013. New genes as drivers of phenotypic evolution. *Nature*
667 *Reviews Genetics* 14:645–660.
668 Cheng CHC, Chen L. 1999. Evolution of an antifreeze glycoprotein. *Nature* 401:443–444.
669 Chevin LM, Lande R, Mace GM. 2010. Adaptation, plasticity, and extinction in a changing
670 environment: towards a predictive theory. *PLOS Biology 8*:e1000357.
671 Christian KA, Morton SR. 1992. Extreme thermophilia in a central Australian ant, *Melophorus*
672 *bagoti*. *Physiological Zoology 65* :885–905.
673 Clémencet J, Cournault L, Odent A, Doums C. 2010. Worker thermal tolerance in the
674 thermophilic ant *Cataglyphis cursor* (Hymenoptera, Formicidae). *Insectes sociaux* 57:11–
675 15.
676 Clusella-Trullas S, Garcia RA, Terblanche JS, Hoffmann AA. 2021: How useful are thermal
677 vulnerability indices? *Trends in Ecology & Evolution 36*:1000–1010
678 Costa G. 2012. *Behavioural adaptations of desert animals*. Springer Science & Business
679 Media.
680 Darras H, Araujo NS, Baudry L, Guiglielmoni N, Lorite P, Marbouty M, Rodriguez F, Arkhipova
681 I, Koszul R, Flot JF, Aron S. 2022. Chromosome-level genome assembly and annotation
682 of two lineages of the ant *Cataglyphis hispanica*: steppingstones towards genomic studies
683 of hybridogenesis and thermal adaptation in desert ants. *Peer Community Journal* 2:e40.
684 Davidson NM, Oshlack A. 2014. Corset: Enabling differential gene expression analysis for de
685 novo assembled transcriptomes. *Genome Biology* 15:1–14.
686 Davidson NM, Hawkins ADK, Oshlack A. 2017. SuperTranscripts: A data driven reference for
687 analysis and visualisation of transcriptomes. *Genome Biology* 18:148.
688 Dhaygude K, Nair A, Johansson H, Wurm Y., Sundström L. 2019. The first draft genomes of
689 the ant *Formica exsecta*, and its *Wolbachia* endosymbiont reveal extensive gene transfer
690 from endosymbiont to host. *BMC Genomics* 20.
691 Emms DM, Kelly S. 2019. OrthoFinder: Phylogenetic orthology inference for comparative
692 genomics. *Genome Biology* 20:238.
693 Evgen'ev MB, Garbuz DG, Shilova VY, Zatsepina OG. 2007. Molecular mechanisms
694 underlying thermal adaptation of xeric animals. *Journal of Biosciences* 32:489–499.
695 Fields PA. 2001. Review: Protein function at thermal extremes: balancing stability and
696 flexibility. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative*
697 *Physiology* 129:417–431.

698 Fields PA, Dong Y, Meng X, Somero GN. 2015. Adaptations of protein structure and function
699 to temperature: there is more than one way to 'skin a cat'. *Journal of Experimental Biology*,
700 218:1801–1811.
701 Gao G, Li J, Li H, Li F, Xu K, Yan G, Chen B, Qiao J, Wu X. 2014. Comparison of the heat
702 stress induced variations in DNA methylation between heat-tolerant and heat-sensitive
703 rape seed seedlings. *Breeding Science* 64:125–133.
704 Grabherr MG, Brian JH, Yassour M, Levin JZ, Thompson DA, Amit I, Adiconis X, Fan L,
705 Raychowdhury R, Zeng Q., et al. 2011. Full-length transcriptome assembly from RNA-seq
706 data without a reference genome. *Nature Biotechnology* 29:644–652.
707 Gurevich A, Saveliev V, Vyahhi N, Tesler G. 2013. QUAST: quality assessment tool for
708 genome assemblies. *Bioinformatics* 29:1072–1075.
709 Hayes JD, Flanagan JU, Jowsey IR. 2005. Glutathione transferases. *Annual Review of*
710 *Pharmacology and Toxicology* 45:51–88.
711 Hazel JR. 1995. Thermal Adaptation in Biological Membranes: Is Homeoviscous Adaptation
712 the Explanation? *Annual Review of Physiology* 57:19–42.
713 Hlina B. 2019. ecotox: analysis of ecotoxicology. R package version 1.4.0. https://CRAN.R-
714 project.org/package=ecotox
715 Hood WG, Tschinkel WR. 1990, Desiccation resistance in arboreal and terrestrial ants.
716 *Physiological Entomology* 15: 23–35.
717 Huang Y, Niu B, Gao Y, Fu L, Li W. 2010. CD-HIT Suite: A web server for clustering and
718 comparing biological sequences. *Bioinformatics* 26:680–682.
719 Jain NK, Roy I. 2008. Effect of trehalose on protein structure. *Protein Science* 18:24–36.
720 Johnson RA. 2000. Habitat segregation based on soil texture and body size in the seed-
721 harvester ants *Pogonomyrmex rugosus* and *P. barbatus*. *Ecological Entomology* 25:403–
722 412.
723 Johnson BR. 2018. Taxonomically restricted genes are fundamental to biology and evolution.
724 *In Frontiers in Genetics* 9:407–407.
725 Johnson DJ, Stahlschmidt ZR. 2020. City limits: Heat tolerance is influenced by body size and
726 hydration state in an urban ant community. *Ecology and Evolution* 10:4944–4955.
727 Jørgensen LB, Ørsted M, Malte H, Wang T, Overgaard J. 2022 Extreme escalation of heat
728 failure rates in ectotherms with global warming. *Nature* 611:93–98.
729 Kaessmann H. 2010. Origins, evolution, and phenotypic impact of new genes. *Genome*
730 *Research* 20:1313–1326.
731 Käfer H, Kovac H, Simov N, Battisti A, Erregger B, Schmidt AKD, Stabentheiner A. 2020.
732 Temperature tolerance and thermal environment of European seed bugs. *Insects* 11:197.

733 Kaspari M, Clay NA, Lucas J, Yanoviak SP, Kay A. 2014. Thermal adaptation generates a
734 diversity of thermal limits in a rainforest ant community. *Global Change Biology* 21:1092–
735 1102.
736 Kokura S, Yoshikawa T, Ohnishi T. 2016. *Hyperthermic oncology from bench to bedside*,
737 Springer, Germany.
738 Laity JJ. 2009. *Deserts and desert environments*. John Wiley & Sons.
739 Lepock JR. 1997. Protein denaturation during heat shock. *Advances in Molecular and Cell*
740 *Biology* 19:223–259.
741 Lou F, Han Z, Gao T. 2019. Transcriptomic responses of two ecologically divergent populations
742 of Japanese mantis shrimp (*Oratosquilla oratoria*) under thermal stress. *Animals 9*:399.
743 Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for
744 RNA-seq data with DESeq2. *Genome Biology* 15:1–34.
745 Marsh AC. 1985. Thermal responses and temperature tolerance in a diurnal desert ant,
746 *Ocymyrmex barbiger*. *Physiological and Biochemical Zoology* 58:629–636.
747 Marsh AC. 1988. Activity patterns of some Namib desert ants. *Journal of Arid Environments,*
748 14:61–73.
749 Martinet B, Dellicour S, Ghisbain G, Przybyla K, Zambra E, Lecocq T, Boustani M, Baghirov
750 R, Michez D, Rasmont P. 2021. Global effects of extreme temperatures on wild
751 bumblebees. *Conservation Biology* 35:1507-1518.
752 Moriya Y, Itoh M, Okuda S, Yoshizawa A, Kanehisa M. 2007. KAAS: an automatic genome
753 annotation and pathway reconstruction server. *Nucleic Acids Research* 35:W182–W185.
754 Musacchia F, Basu S, Petrosino G, Salvemini M, Sanges R. 2015. Annocript: A flexible pipeline
755 for the annotation of transcriptomes able to identify putative long noncoding RNAs.
756 *Bioinformatics* 31:2199–2201.
757 Nguyen AD, Gotelli NJ, Cahan SH. 2016. The evolution of heat shock protein sequences, cis-
758 regulatory elements, and expression profiles in the eusocial Hymenoptera. *BMC*
759 *Evolutionary Biology 16*:15.
760 Pandian BA, Sathishraj R, Djanaguiraman M, Prasad PVV, Jugulam M. 2020. Role of
761 Cytochrome P450 enzymes in plant stress response. *Antioxidants* 9:454.
762 Patro R, Duggal G, Love M, Irizarry RA, Kingsford C. 2017. Salmon provides fast and bias-
763 aware quantification of transcript expression. *Nature Methods* 14:417–419.
764 Perez R, Aron S. 2020. Adaptations to thermal stress in social insects: recent advances and
765 future directions. *Biological Reviews* 95:1535–1553.
766 Perez R, Araujo NS, Defrance M., Aron S. 2021. Molecular adaptations to heat stress in the
767 thermophilic ant genus *Cataglyphis*. *Molecular Ecology* 30:5503–5516.
768 Richter K, Haslbeck M, Buchner J. 2010. The heat shock response: life on the verge of death.
769 *Molecular Cell* 40:253–266.

770 Robinson MD, McCarthy DJ, Smyth GK. 2009. EdgeR: A bioconductor package for differential
771 expression analysis of digital gene expression data. *Bioinformatics* 26:139–140.
772 Roeder KA, Roeder DV, Bujan J. 2021. Ant thermal tolerance: a review of methods,
773 hypotheses, and sources of variation. *Annals of the Entomological Society of America*
774 114:459–469
775 Sælensminde G, Halskau Ø, Jonassen I. 2009. Amino acid contacts in proteins adapted to
776 different temperatures: hydrophobic interactions and surface charges play a key role.
777 *Extremophiles* 13:11.
778 Sala OE, Chapin FS, Armesto JJ, Berlow E, Bloomfield J, Dirzo R, Huber-Sanwald E,
779 Huenneke LF, Jackson RB, Kinzig A, et al. 2000. Global biodiversity scenarios for the year
780 2100. *Science* 287:1770–1774.
781 Shi N, Tsai C, Camino F, Bernard GD, Yu N, Wehner R. 2015. Keeping cool: enhanced optical
782 reflection and radiative heat dissipation in Saharan silver ants. *Science* 349:298–301.
783 Simão FA, Waterhouse RM, Ioannidis P, Kriventseva EV, Zdobnov EM. 2015. BUSCO:
784 Assessing genome assembly and annotation completeness with single-copy orthologs.
785 *Bioinformatics* 31:3210–3212.
786 Simola DF, Wissler L, Donahue G, Waterhouse RM, Helmkampf M, Roux J, Nygaard S,
787 Glastad KM, Hagen DE, Viljakainen L, et al. 2021. Social insect genomes exhibit dramatic
788 evolution in gene composition and regulation while preserving regulatory features linked
789 to sociality. *Genome Research* 23:1235–1247.
790 Solis DR, Bueno OC. 2012. Thermal tolerances of three tramp ant species (Hymenoptera:
791 Formicidae). *Sociobiology* 59:1.
792 Sommer S, Wehner R. 2012. Leg allometry in ants: extreme long-leggedness in thermophilic
793 species. *Arthropod Structure and Development* 41:71–77.
794 Sunday JM, Bates AE, Dulvy NK. 2011. Global analysis of thermal tolerance and latitude in
795 ectotherms. *Proceedings of the Royal Society B* 278:1823–1830.
796 Sunday JM, Bates AE, Kearney MR, Colwell RK, Dulvy NK, Longino JT, Huey RB. 2014.
797 Thermal-safety margins and the necessity of thermoregulatory behavior across latitude
798 and elevation. *Proceedings of the National Academy of Sciences of the United States of*
799 *America* 111:5610–5615.
800 Supek F, Bošnjak M, Škunca N, Šmuc T. 2011. REVIGO summa-rizes and visualizes long lists
801 of gene ontology terms edited by C. Gibas. *Plos One* 6:e21800.
802 Suzek BE, Wang Y, Huang H, McGarvey PB, Wu CH. 2015. UniRef clusters: A comprehensive
803 and scalable alternative for improving sequence similarity searches. *Bioinformatics* 31:
804 926–932.
805 Taylor JS, Raes J. 2004. Duplication and divergence: The evolution of new genes and old
806 ideas. *Annual Review of Genetics* 38:615–643.

807 Turner R, Lightort JR, Turner JS. 2000. Critical thermal maximum of tile African ant,
808 *Ocymyrmex* sp.: possibility of a new record prompts a review of methodologies.
809 *Comparative Biochemistry and Physiology B* 126:S1–S108.
810 Valles SM, Strong CA, Buss EA, Oi DH. 2012. Non-enzymatic hydrolysis of RNA in workers of
811 the ant *Nylanderia pubens*. *Journal of Insect Science* 12:146.
812 Wheeler MW, Park RM, Bailey AJ. 2006. Comparing median lethal concentration values using
813 confidence interval overlap or ratio tests. *Environmental Toxicology and Chemistry*
814 *25*:1441–1444.
815 Wehner R. 2020. Desert Navigator. *The Journey of an Ant*. Harvard University Press,
816 Cambridge.
817 Wehner R, Wehner S. 2011. Parallel evolution of thermophilia: daily and seasonal foraging
818 patterns of heat-adapted desert ants: *Cataglyphis* and *Ocymyrmex* species. *Physiological*
819 *Entomology* 36:271–281.
820 Wehner R, Marsh AC, Wehner S. 1992. Desert ants on a thermal tightrope. *Nature* 357:586–
821 587.
822 Wendt CF, Verble-Pearson R. 2016. Critical thermal maxima and body size positively correlate
823 in red imported fire ants, *Solenopsis invicta*. *The Southwestern Naturalist* 61:79–83.
824 Werner MS, Sieriebriennikov B, Prabh N, Loschko T, Lanz C, Sommer RJ. 2018. Young genes
825 have distinct gene structure, epigenetic profiles, and transcriptional regulation. *Genome*
826 *Research* 28:1675–1687.
827 Willot Q, Simonis P, Vigneron JP, Aron S. 2016. Total internal reflection accounts for the bright
828 color of the saharan silver ant. *PLoS One* 11:e0152325.
829 Willot Q, Gueydan C, Aron S. 2017. Proteome stability, heat hardening, and heat-shock protein
830 expression profiles in *Cataglyphis* desert ants. *Journal of Experimental Biology* 220:
831 1721–1728.
832 Willot Q, Mardulyn P, Defrance M, Gueydan C, Aron S. 2018. Molecular chaperoning helps
833 safeguarding mitochondrial integrity and motor functions in the Sahara silver ant
834 *Cataglyphis bombycina*. *Scientific Reports* 8:9220.
835 Wissler L, Gadau J, Simola DF, Helmkampf M, Bornberg-Bauer E. 2013. Mechanisms and
836 dynamics of orphan gene emergence in insect genomes. *Genome Biology and Evolution*
837 *5* :439–445.
838 Woods HA, Dillon ME, Pincebourde S. 2015. The roles of microclimatic diversity and of
839 behavior in mediating the responses of ectotherms to climate change. *Journal of Thermal*
840 *Biology* 54:86–97.
841 Yu D, Zhang Z, Shen Z, Zhang C, Liu H. 2018. Regional differences in thermal adaptation of
842 a cold-water fish *Rhynchocypris oxycephalus* revealed by thermal tolerance and
843 transcriptomic responses. *Scientific Reports* 8:11703.

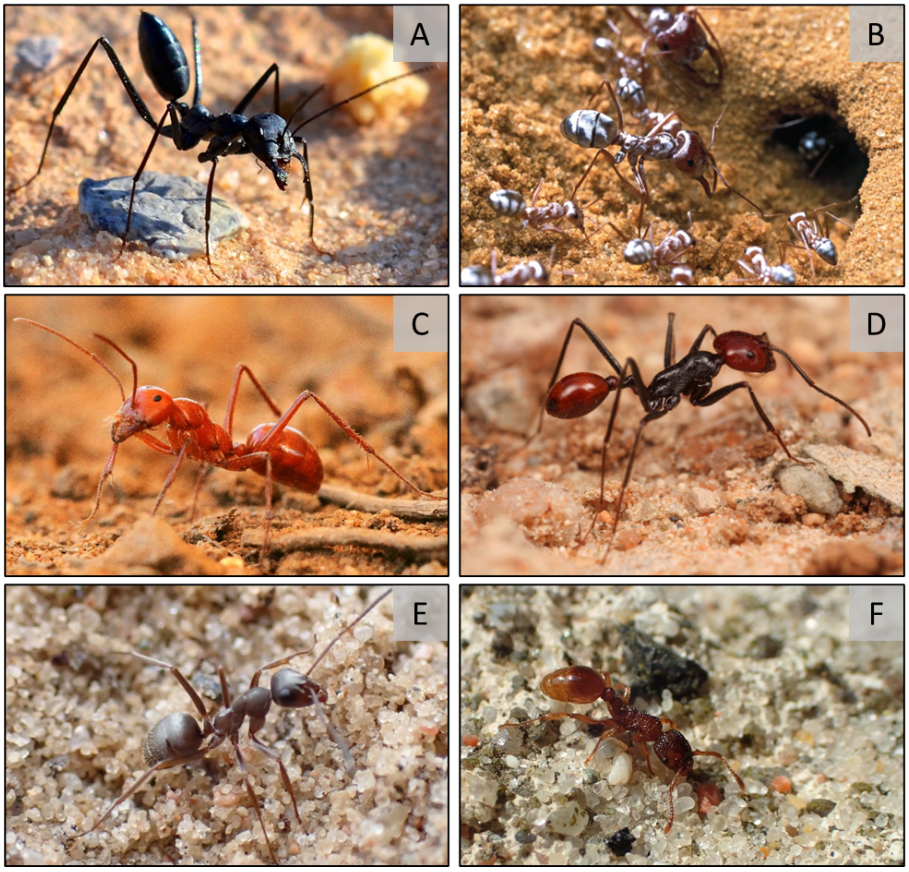

Figure 1. Workers of the ant species: (A) *Cataglyphis holgerseni* by A. Kuhn; (B) *C. bombycina* by Q. Willot; (C) *Melophorus bagoti* by A. Wystrach; (D) *Ocymyrmex sp.* by R. Duncan; (E) *Formica sp.* by Q. Willot; and (F) *Myrmica sabuleti* by Q. Willot.

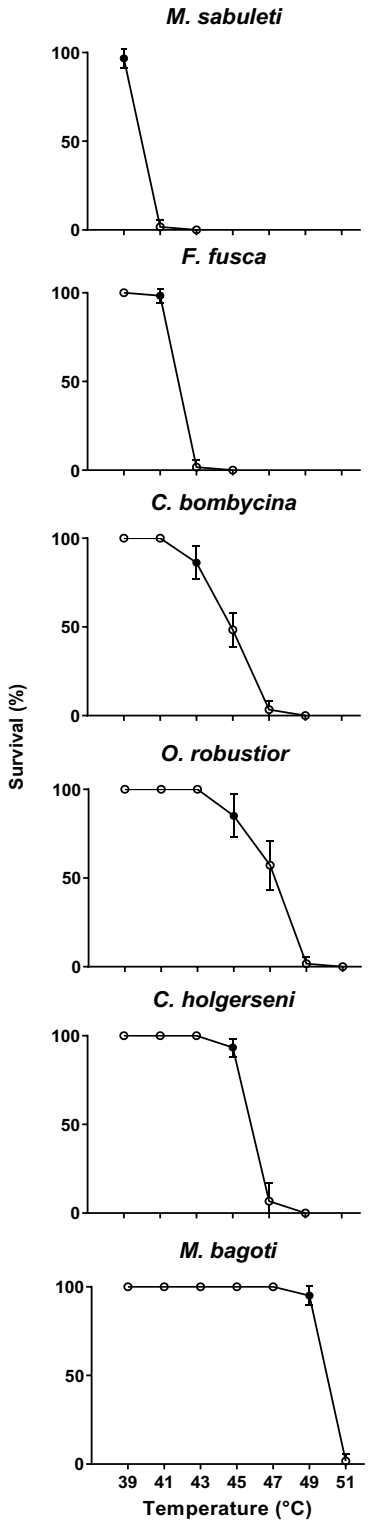

Figure 2. Percent survival of ant workers during heat-stress assays. The ants were exposed to increasing temperatures (39°C, 41°C, 43°C, 45°C, 47°C, 49°C or 51°C) for 3 hours; their percent survival was then measured. Workers were categorized as moribund when reaching muscular paralysis. For each species, UTL is indicated by a filled circle (•).

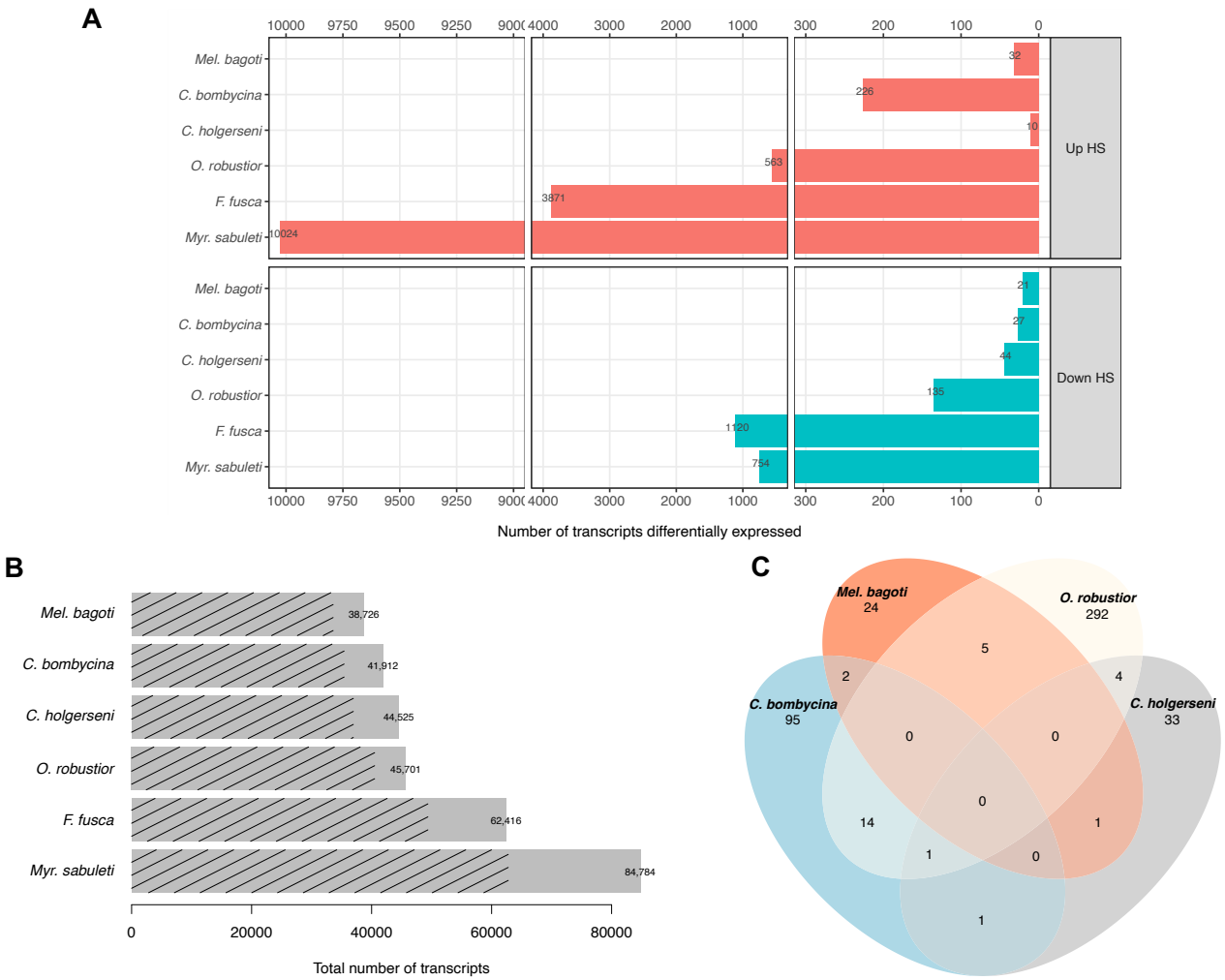

Figure 3. Transcriptomic analyses summary. **A** – Number of differentially expressed transcripts between heat stressed and non-heat stressed conditions for each species tested. Up HS transcripts refers to transcripts up regulated in the heat stressed samples when compared to the non-heat stressed samples, while Down HS refers to transcripts down regulated in heat stressed samples (i.e., up regulated in non-heat stressed samples in comparison to heat-stressed ones). **B** – Reference transcriptome sizes. Dashed lines show the proportion of transcripts with at least one significant blast hit. **C** – Number of transcripts differentially expressed and annotated in common among desert species. Only the overlap between *C. bombycina* and *O. robustior* was significant at $p < 0.01$ random sampling test (Table S3).

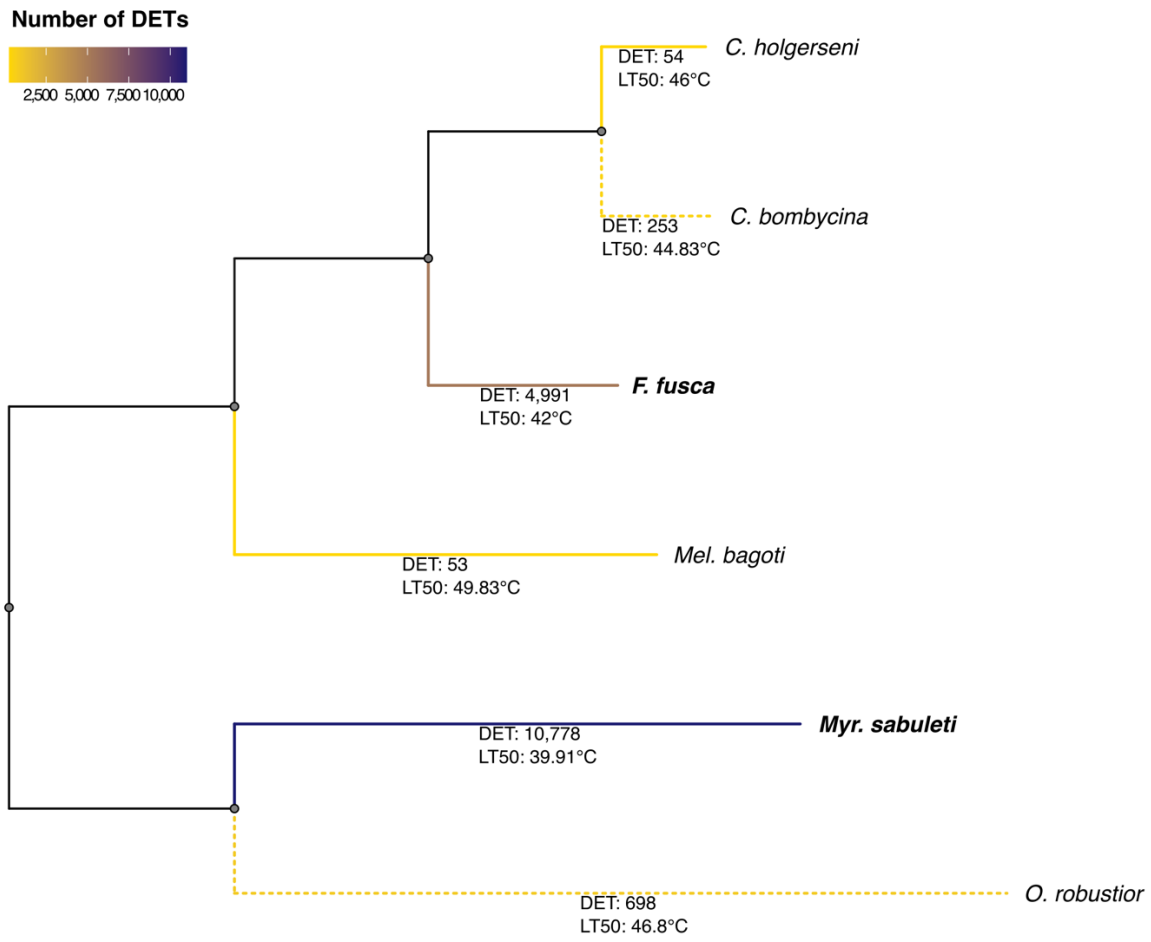

Figure 4. Phylogenetic tree of the studied ant species estimated using orthologous proteins. Branch colours corresponds to the number of differentially expressed transcripts (DET) identified for that species between heat stressed and non-heat stressed samples. The exact number of DET is reported bellow each species branch along with the LT50. Grey lines represent internal nodes with no values. Dotted lines indicate species showing gradual decrease in survival ratio within a six-degree temperature interval while straight lines refer to species whose survival decline from 100 to 0% within a smaller temperature range of two degrees. Ant species from temperate areas are shown in bold.

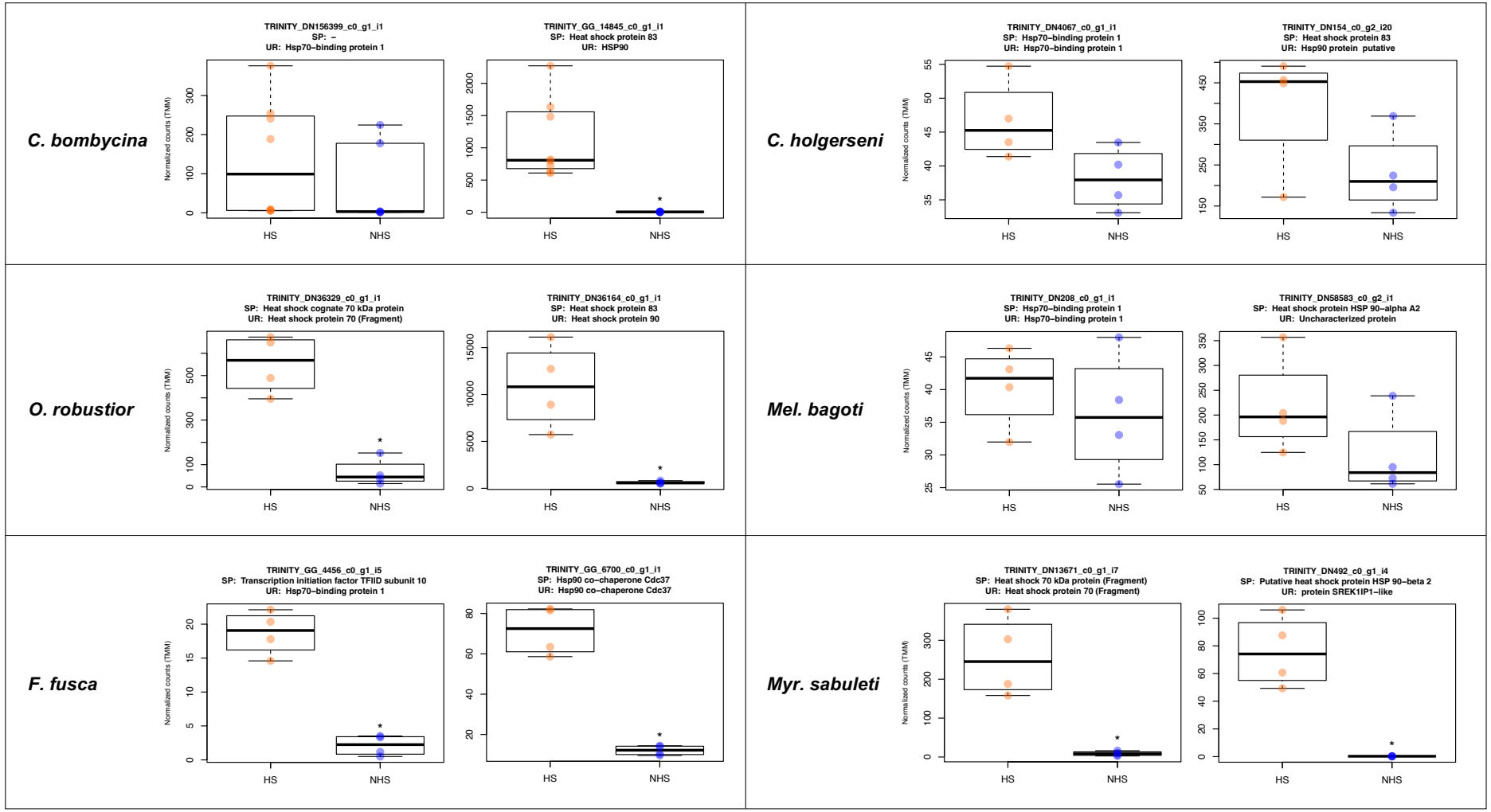

Figure 5. Expression of heat stress proteins 70 and 90 related transcripts in heat-stressed and non-heat stressed treatments. Expression levels shown as TMM normalized read counts. Transcripts shown represent one related transcript from each HSP that was either differentially expressed or had highest expression in the referred species. HS – samples heat stressed; NHS – non-heat stressed control samples; SP – UniProtKB/Swiss-Prot annotation; UR – UniRef90 database annotation. * Indicates the transcript is significantly differentially regulated between treatment conditions ($\log_2$-fold change ≥ 2 between treatments and FDR ≤ 1e-3).

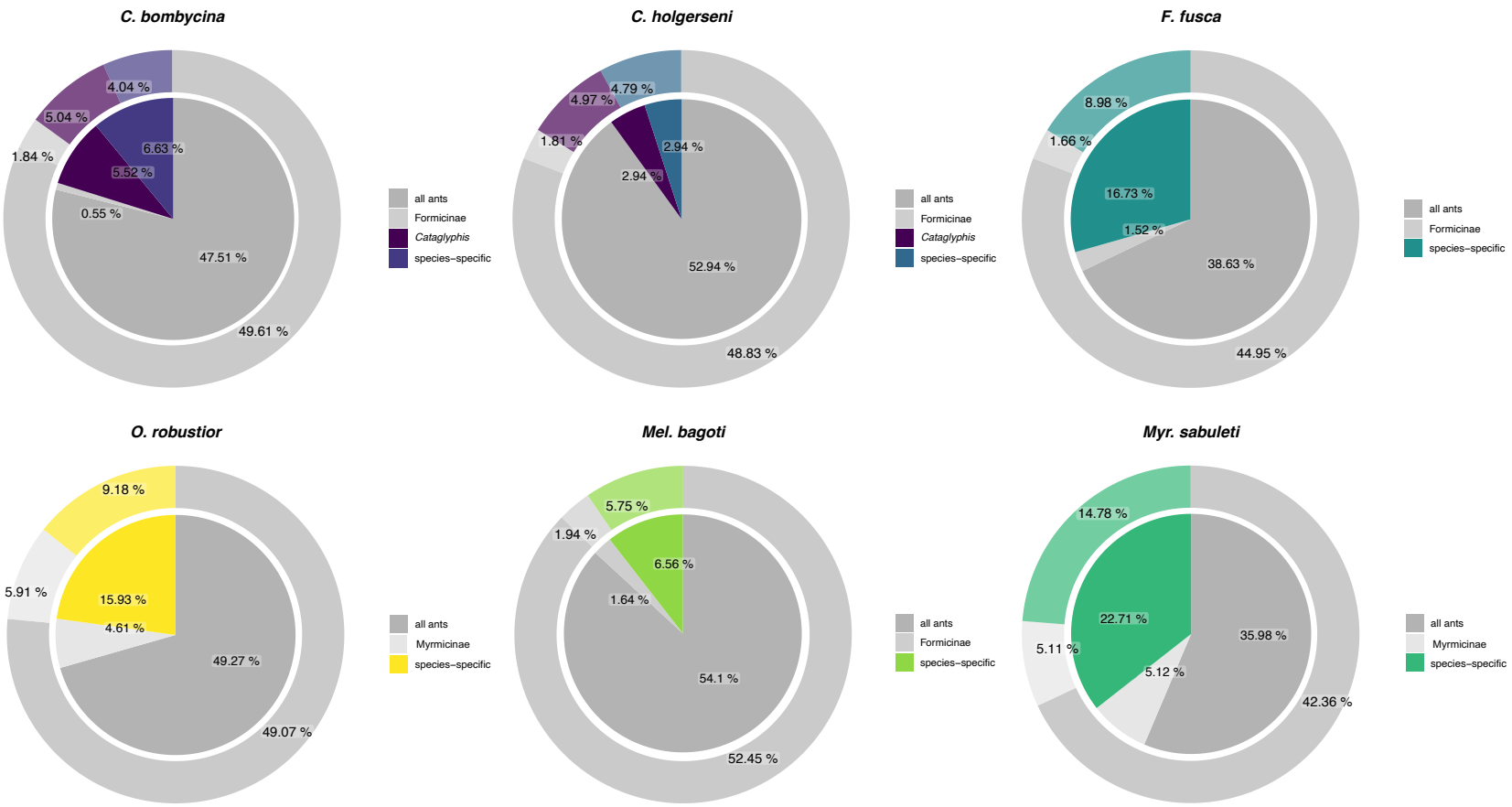

Figure 6. Relative proportion of transcripts in different orthologous categories. Colours highlight lineage specific categories while grey shades illustrate taxonomically conserved orthologous categories. Inner circles represent the proportion of transcripts in each category among the set of differentially expressed transcripts while outer circles show these proportions in the full transcriptome for comparison. Differences between the proportions observed in *F. fusca*, *Myr. sabuleti* and *O. robustior* are significant based on alpha level of .01.