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RESEARCH ARTICLE

Proteome stability, heat hardening and heat-shock protein expression profiles in *Cataglyphis* desert ants

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ABSTRACT

In ectotherms, high temperatures impose physical limits, impeding activity. Exposure to high heat levels causes various deleterious and lethal effects, including protein misfolding and denaturation. Thermophilic ectotherms have evolved various ways to increase macromolecular stability and cope with elevated body temperatures; these include the high constitutive expression of molecular chaperones. In this study, we investigated the effect of moderate to severe heat shock (37–45°C) on survival, heat hardening, protein damage and the expression of five heat tolerance-related genes (*hsc70-4 h1*, *hsc70-4 h2*, *hsp83*, *hsc70-5* and *hsf1)* in two closely related *Cataglyphis* ants that occur in distinct habitats. Our results show that the highly thermophilic Sahara ant *Cataglyphis bombycina* constitutively expresses HSC70 at higher levels, but has lower induced expression of heat tolerance-related genes in response to heat shock, as compared with the more mesophilic *Cataglyphis mauritanica* found in the Atlas Mountains. As a result, *C. bombycina* demonstrates increased protein stability when exposed to acute heat stress but is less disposed to acquiring induced thermotolerance via heat hardening. These results provide further insight into the evolutionary plasticity of the *hsp* gene expression system and subsequent physiological adaptations in thermophilous desert insects to adapt to harsh environmental conditions.

KEY WORDS: Heat stress, Molecular chaperone, Heat shock response

INTRODUCTION

Protein structural stability is paramount to the maintenance of cell integrity and is highly dependent on temperature. Proteotoxic stressors, such as heat shock, can cause proteins to misfold and denature, resulting in their aggregation and a subsequent loss of biological function and an impairment of normal metabolic processes (Sørensen et al., 2003; Mayer, 2010). Accordingly, the heat-shock response (HSR) was selected for early on in evolutionary time and has been widely conserved across taxa (Moseley, 1997; Feder and Hofman, 1999). It includes the production of molecular chaperones – such as heat-shock proteins (HSPs) – that are involved in the assembly, folding and proper translocation of cellular proteins (Sørensen et al., 2003; Moseley, 1997; Feder and Hofman, 1999). Expression of HSPs depends on the activation of transcriptional heat-shock factors (HSFs). HSF1 trimerises during activation and binds to specific *cis*-regulatory elements in the genome – known as heat-shock elements (HSEs) – and thus induces the expression of target genes, including molecular chaperones (Sarge et al., 1993; Zatsepina et al., 2000). The constitutive expression of HSPs and their relative levels of induced expression under stressful conditions are associated with an organism's adaptive capacity to tolerate thermal pressures (Evgen'ev et al., 2014). Comparisons among species from contrasting environments have shown that thermophilic species usually have higher constitutive levels of HSPs than do their mesophilic relatives. However, their relative induced expression of HSPs is lower in response to heat stress (Rinehart et al., 2006; Dong et al., 2008; Clack and Peck, 2009; Evgen'ev et al., 2014). Moreover, in thermophiles, HSF activation and the resulting induction of HSP synthesis occur at higher temperatures. Ultimately, proteins of thermophiles are more stable than those of mesophiles, whether as a result of structural changes or as a consequence of large amounts of constitutive HSPs (Evgen'ev et al., 2014). It has been shown that both the duplication of *hsp* genes and shifts in HSE arrangement and position allow for greater HSR functional plasticity, helping organisms adopt the best-suited response to environmental heat stress (Bettencourt and Feder, 2001; Tian et al., 2010; Nguyen et al., 2016). The use of this system is well developed in ectotherms, which cannot regulate their body temperatures and whose metabolic processes and stability therefore depend on microclimatic conditions (Hazel, 1995; Lin and Somero, 1995; Willmer et al., 2000). Consequently, ectotherms are interesting models with which to explore HSR patterns under conditions of thermal stress (Evgen'ev et al., 2014).

Cataglyphis desert ants present a unique opportunity for investigating the HSR in heat-tolerant terrestrial ectotherms. All *Cataglyphis* species inhabit arid lands or deserts – they are found in the Sahara, the Near East, the Middle East, the Arabian Peninsula and Central Asia (Lenoir et al., 2009; Boulay et al., 2017). In contrast to many other desert-adapted species, which tend to escape the heat by being nocturnal or crepuscular, *Cataglyphis* ants take advantage of their high thermal tolerance to remain active even during the hottest parts of the day: most are scavengers and collect the bodies of less-tolerant, heat-stricken arthropods (Harkness and Wehner, 1977; Wehner et al., 1992). These ants are generally equipped with long legs, which increase the distance between their bodies and the soil; this trait reduces exposure of the body core to high ground temperatures, thus improving body cooling, and also enhances running velocity (Marsh, 1985; Sommer and Wehner, 2012). Previous studies have shown that foragers of the Saharan silver ant, *Cataglyphis bombycina*, produce what was thought to be HSP70 before emerging from the nest, thereby bypassing the need to acclimate to sudden heat exposure (Gehring and Wehner, 1995). However, more recently, it was discovered that the primary heat-induced gene *hsp70* has been completely lost in Hymenoptera; instead, two *hsc70-4* paralogues are expressed (Nguyen et al., 2016). At present, the ability to express other HSPs and to induce gene expression in response to heat stress remains completely unexplored in *Cataglyphis*.

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Received 29 November 2016; Accepted 17 February 2017

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In this study, we compared the HSRs of two closely related *Cataglyphis* species – *C. bombycina* and *C. mauritanica* – found in natural habitats that contrast in mean annual temperature and temperature seasonality (Fig. 1). *Cataglyphis bombycina* is found in warm deserts, which are characterised by extremely high summer temperatures and warm winters. This species has one of the highest critical thermal maximum values observed among terrestrial organisms (CT_{max}=53.6°C; Hoffmann et al., 2013). Workers are active during the hottest parts of the day, when air temperatures may exceed 50°C (Wehner et al., 1992). Their lower limit for foraging activity is influenced by the presence of their main lizard predator, *Acanthodactylus dumerili*, which retreats to its underground burrow when temperatures becomes unbearable. Workers are thus constrained to forage within a narrow thermal window (46.5–53.6°C). *Cataglyphis mauritanica*, in contrast, lives in the Atlas Mountains, which are characterised by a cold semi-arid climate, with warm summers and cold winters. This species experiences heat stress during the summer, and their habitat exhibits extreme diurnal and seasonal temperature fluctuations that are associated with altitude (Dillon et al., 2006; Peel et al., 2007). As a result, when foraging, *C. mauritanica* workers probably face lower but much more variable temperatures than do *C. bombycina* workers.

First, we compared the species' general heat tolerance and their ability to induce thermotolerance via heat hardening (i.e. the synthesis of thermoprotective molecules following mild heat stress; Feder and Hofmann, 1999) by exposing workers to sublethal and lethal heat shocks. Second, we quantified *in vivo* protein damage resulting from heat shock by examining protein aggregation levels. Third, we compared the levels of constitutively expressed HSC70 between the two species. Fourth, we characterised the induced expression of several *hsp* genes – *hsf1, hsc70-4h*, *hsp83* and *hsc70-5* – known to be associated with HSR, heat tolerance and protein folding (Sarge et al., 1993; Feder and Hofmann, 1999; Nguyen et al., 2016). Based on current knowledge regarding the molecular mechanisms underlying adaptations to extreme environments (Evgen'ev et al., 2014), we predicted that the more thermophilic species, *C. bombycina*, would constitutively express molecular chaperones at higher levels than would the more mesophilic species, *C. mauritanica*. Similarly, we predicted that *C. bombycina* would show lower levels of induced *hsp* gene expression, reduced use of heat hardening to increase thermal tolerance, and lower levels of heat-shock-related protein damage.

MATERIALS AND METHODS

Field sampling and laboratory rearing

Colonies of *C. bombycina* Roger 1859 were collected near Zagora (30°19′56″N, 5°50′18″W), located in the Draa Valley in southern Morocco. Zagora is characterised by a warm desert climate; it experiences extremely high summer temperatures from early spring to late autumn and warm winters (mean summer temperature 33.4°C, altitude 724 m). Colonies of *C. mauritanica* (Emery 1906) were collected in Ifrane National Park (33°33′22″N, 5°14′15″W), located in the Atlas Mountains of Morocco. Ifrane is characterised by a cold semi-arid climate; it experiences warm summers and cold winters (mean summer temperature 22.7°C, altitude 1730 m; Peel et al., 2007). Colonies were kept in 30×40×10 mm plastic boxes with Fluon®-coated sides and a thin layer of clean sand on the bottom. For nesting, they were given 16×150 mm glass test tubes; the bottoms contained water, which ants could access via a moist cotton plug. The ant colonies were reared under constant environmental conditions: a temperature of 25°C, a 12 h:12 h light:dark cycle and 60% relative humidity. They were provided with a sugar solution *ad libitum* and sliced mealworms twice a week. They were kept under laboratory conditions for at least 2 months prior to experimentation. All the experiments were performed on workers hatched and raised in the laboratory (i.e. from the egg to adult stage).

Fig. 1. *Cataglyphis bombycina* (left) and *Cataglyphis mauritanica* (right) workers. *Cataglyphis bombycina* lives in the Sahara desert, while *C. mauritanica* is found in the Atlas Mountains; *C. bombycina* is the more thermophilic of the two.

Thermal tolerance and heat hardening

General thermal tolerance was determined using heat-stress experiments. Three temperature treatments were used – 37, 40 and 45°C – which corresponded to mild, medium and severe heat stress. Previous studies (Gehring and Wehner, 1995) showed that HSC70 synthesis continues up to 45°C in the silver ant. For each treatment, three to six groups of 15 workers were used per species. Worker groups were placed in 50 ml glass vials containing moist cotton balls. The vials were then submerged in a digital water bath (SW22, Julabo GmbH, Seelbach, Germany); the temperature inside the vials was monitored using 0.075 mm diameter thermocouples [Type K Thermocouple (Chromel/Alumel), RS Components Ltd, Glasgow, UK] connected to a digital thermometer (RS Pro RS52 Digital Thermometer, RS Components Ltd). The ants were exposed to the treatment for 3 h. This duration of exposure was previously used to induce a significant HSR in *C. bombycina* (Gehring and Wehner, 1995). The species' use of heat hardening was assessed by pre-exposing workers to mild heat stress (37°C) for 2 h before they experienced severe heat stress (45°C) for 3 h. In both the thermal-tolerance and heat-hardening experiments, ants that could still move after the treatments were considered to be alive. We used worker survival rates as proxies for levels of thermal tolerance and heat hardening.

Protein damage

To examine the protein damage caused by heat stress, we compared protein aggregation levels between control workers, which spent 3 h at 25°C, and heat-shocked workers, which spent 3 h at 45°C; the workers were immediately flash frozen following treatment. Nine groups of six individuals were used per treatment per species. Protein aggregates were extracted and purified as per Chen et al. (2002) and Rinehart et al. (2006). Briefly, workers were pooled and placed in a chilled extraction buffer consisting of 0.1% Triton X-100, 60 mmol l^{-1} PIPES, 1 mmol l^{-1} EDTA, 1 mmol l^{-1} EGTA, 100 mmol l^{-1} NaCl, 0.5 mmol l^{-1} PMSF, 0.75 mg l^{-1} leupeptin and 0.1 mmol l^{-1} DL-DTT (Sigma-Aldrich Chemie GmbH, Munich, Germany). They were then homogenised for 3 min at maximum speed in a mixer mill (MM 301, Retsch GmbH, Haan, Germany) with 2.8 mm stainless steel beads. The resulting homogenate was filtered through a 21-gauge needle (Sterican®, Braun, Kronberg, Germany) to remove large pieces of cuticle and organs. The filtrate was then centrifuged at 680 ***g*** for 10 min at 4°C to pellet nuclei and large cell fragments. The supernatant was centrifuged at 35,000 ***g*** for 14 min at 4°C (Optima™ L-70K

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Ultracentrifuge, Beckman Coulter, Chaska, MN, USA) to separate the Triton-soluble fraction from the Triton-insoluble fraction (Chen et al., 2002). To purify the protein aggregates, the Triton-insoluble fraction was twice suspended, then sonicated and pelleted at 17,000 ***g*** for 30 min at 4°C. The resulting pellet was resuspended, sonicated and pelleted at 5000 ***g*** for 30 min at 4°C. Then, the pelleted protein aggregates were resuspended and assayed for protein content. Protein concentrations were determined using the Bradford procedure (BioRad, Hercules, CA, USA) and BSA as a standard (Rinehart et al., 2006).

Constitutive levels of HSC70

Constitutive levels of HSC70 were estimated by western blotting. We extracted total protein from 20 mg of randomly selected control and heat-shocked workers (see above) per species. Protein concentrations were determined using the Bradford procedure (BioRad). A 15 µg sample of the extract was mixed with 6-fold concentrated Laemmli buffer (1% SDS, 5% β-mercaptoethanol and 10% glycerin; Sigma-Aldrich Chemie GmbH) in a 1:6 ratio and kept at 95°C for 5 min. Samples were then diluted in Laemmli buffer and analysed in duplicate by SDS-PAGE on a 10% gel. The gel was stained with Coomassie Blue and the total signal for each lane was quantified using a CCD camera, which established the control loading values for each sample. Proteins were then electrotransferred onto an Amersham Protran 0.45 µm nitrocellulose blotting membrane (GE Healthcare Life Sciences, Little Chalfont, UK). Skimmed milk (1.5%) was used for blocking (1 h, 20°C) and to dilute the antibodies. The membrane was washed three times with TBS-T buffer (10 mmol l^{-1} Tris, 150 mmol l^{-1} NaCl and 2% Tween-20) and incubated overnight at 4°C with an anti-HSP70 monoclonal mouse antibody (1/2000 dilution; clone BRM-22, Sigma-Aldrich, Rehovot, Israel). Secondary antibodies (1/10,000 dilution; anti-mouse IgG peroxidase, Sigma-Aldrich) were then added, and the solution was left at 20°C for 2 h. Signal strength was quantified using an Odyssey® FC Imaging System (Li-Cor® Bioscience, Lincoln, NE, USA).

Heat stress and gene upregulation

To examine the species' ability to induce the expression of the target *hsp* genes in response to heat stress, total RNA was extracted from control workers, heat-shocked workers (see above) or heat-shocked workers after 1 h respite time at 25°C. We used three groups of six randomly selected workers per treatment per species. Samples were pooled by treatment and placed in 1 ml of chilled Trizol reagent (Invitrogen, Carlsbad, CA, USA). They were then homogenised for 3 min at maximum speed in a mixer mill (MM 301, Retsch GmbH) with 2.8 mm zirconium oxide beads (Bertin Technologies, Montigny-le-Bretonneux, France). Total RNA was extracted according to the manufacturer's instructions. RNA was quantified with a NanoDrop 1000 spectrophotometer (ThermoFisher Scientific Bvba, Gent, Belgium). The 260 nm/280 nm absorbance ratios for the samples were between 1.8 and 2.0, indicating sufficient RNA purity.

After DNase treatment (amplification-grade DNAse I, ThermoFisher Scientific, Carlsbad, CA, USA), 500 ng of total RNA was retrotranscribed using a RT-mix kit (SuperScript® III Reverse Transcriptase, ThermoFisher Scientific). Primers were designed according to quality guidelines for qPCR to detect specific target genes (Thornton and Basu, 2011; Table S1). Quantitative PCR (qPCR) was performed using an ABI StepOnePlus Real-Time PCR System (ThermoFisher Scientific). We used 20 µl reaction volumes containing 2 ng of template cDNA, 8 nmol l^{-1} of total primer and 10 µl of SYBR® (Premix Ex Taq™ II, Takara Bio USA Inc., Mountain View, CA, USA). The following temperature program was used: an initial incubation step at 95°C for 2 min, then 40 cycles at 95°C for 15 s, and finally an annealing step followed by an extension step, each at 60°C for 60 s. Melt curve analysis confirmed the presence of a single amplicon. Control qPCR reactions were performed using RNA extract as a template to confirm the absence of genomic DNA contamination. Relative changes in gene expression were calculated using the ΔΔCT method (Livak and Schmittgen, 2001) and Rest software v.2.0.13 (Pfaffl et al., 2002). We screened for several commonly used housekeeping genes; the gene set we used for standardisation [i.e. *β-actin* and *eukaryotic elongation factor-1* (*eEF-1*)] was determined using both Normfinder (Andersen et al., 2004) and GeneNorm (Vandesompele et al., 2002).

Statistical analyses

Survival rates in the thermal tolerance and heat-hardening experiments, as well as levels of protein aggregation in response to heat stress, were compared using one-way analyses of variance (ANOVA). Multiple comparisons among pairs of means were carried out using Tukey *post hoc* tests. Because 100% of *C. mauritanica* workers survived in the 37 and 40°C treatments (meaning residuals did not meet the assumption of normality; see Results), we also tested for an association between survival and temperature (37, 40 and 45°C) for each species using a non-parametric Spearman's rank correlation test. Before analysing the gene expression patterns, the relative expression values were log transformed to meet assumptions of normality. The significance of treatment on gene upregulation in each species was tested using ANOVA followed by Tukey *post hoc* tests. Relative changes in gene expression between species were analysed with Student's *t*-tests.

RESULTS

Thermal tolerance and heat hardening

To assess general thermal tolerance, we measured the survival rates of *C. bombycina* and *C. mauritanica* workers exposed to mild (37°C), medium (40°C) and severe (45°C) heat stress (Fig. 2). *Cataglyphis bombycina* workers showed a slight, but non-

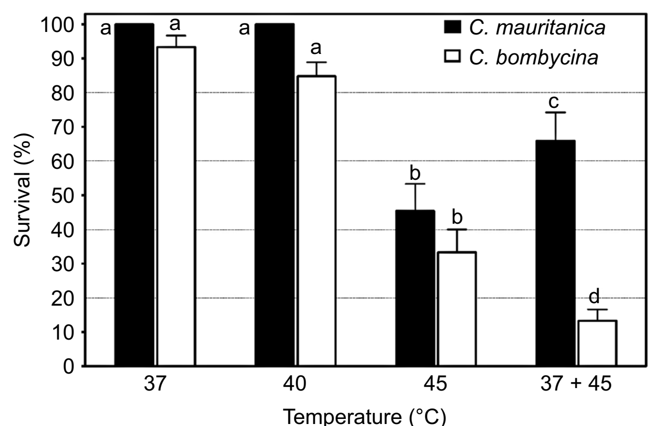

Fig. 2. Survival rate of *C. mauritanica* and *C. bombycina* workers exposed to various temperature regimes. Mean±s.d. percentage survival in three to six groups of 15 individuals each. Exposure to 37, 40 or 45°C for 3 h did not cause significant differences in mortality rates between species. Survival decreased dramatically after a 3 h, 45°C heat stress for both species. A mild heat hardening of 2 h at 37°C followed by heat stress of 3 h at 45°C significantly increased survival in *C. mauritanica* while it decreased survival in *C. bombycina*. Different lowercase letters indicate significant differences between treatments (one-way ANOVA and Tukey *post hoc* test, $P<0.05$).

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significant decrease in survival rate in response to mild and medium heat stress (mean±s.d. survival rate: 93±4.4% and 88.7±7.7%, respectively), while all the *C. mauritanica* workers in these heat-stress treatments survived (mean±s.d. survival rate: 100±0% for both temperatures). However, severe heat stress resulted in a dramatic decrease in worker survival in both species (mean±s.d. survival rate for *C. bombycina*: 33±8.9% and *C. mauritanica*: 45.5±13.51%; one-way ANOVA: $F_{7,40}$=69.91, P<0.0001; Tukey *post hoc* test, P<0.05). Consistent with this result, Spearman's rank correlation tests showed a significant negative association between survival rate and temperature within each species (*C. bombycina*: r_s=−0.774, n=12, P=0.003; *C. mauritanica*: r_s=−0.868, n=10, P=0.002). Worker survival did not differ between species (P>0.05).

There was no evidence of heat hardening in *C. bombycina*. Rather, treatment of workers appeared to decrease survival (13.3±4.4%; P<0.05). In contrast, in *C. mauritanica*, treated workers had significantly higher survival rates (control: 45.5±13.51% versus treated: 65.9±10.6%; P<0.05), suggesting heat hardening occurred in this species (Fig. 2).

Protein damage

We examined the protein damage resulting from heat stress by comparing protein aggregation levels between control and heat-shocked workers. Control workers had low levels of protein aggregation (mean±s.d., *C. bombycina*: 1.39±1.04 ng μg^{-1} total protein extract; *C. mauritanica*: 1.42±0.54 ng μg^{-1}; Fig. 3). When exposed to heat stress, both species showed increased levels of protein aggregation (*C. bombycina*: 5.01±2.24 ng μg^{-1}; *C. mauritanica*: 9.53±2.37 ng μg^{-1}; $F_{3,56}$=23.23, P<0.0001; Tukey *post hoc* test: P<0.05). Protein aggregation levels in heat-shocked workers were significantly higher in *C. mauritanica* than in *C. bombycina* (P<0.001).

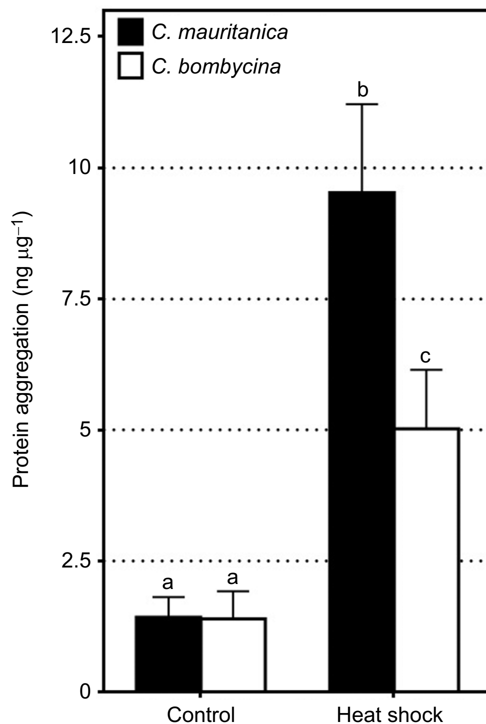

Fig. 3. Protein aggregation after heat stress in *C. mauritanica* and *C. bombycina* workers. Mean±s.d. protein aggregation (ng μg^{-1} total protein) in nine groups of six individuals each, exposed to heat shock (45°C) or control (25°C) conditions for 3 h. Both species experienced an increase in protein aggregation after heat stress; however, the increment was significantly higher in *C. mauritanica* than in *C. bombycina*. Different lowercase letters indicate a significant difference between treatments (one-way ANOVA and Tukey *post hoc* test, P<0.05).

Constitutive levels of HSC70

The concentration of HSC70 in the protein extract was assessed by western blotting and corrected for the total amount of protein loaded per sample using Coomassie Blue signal strength. The results are expressed as the ratio of HSC70 to total protein. Higher constitutive levels of HSC70 were detected in *C. bombycina* than in *C. mauritanica* (Fig. 4).

Heat stress and gene upregulation

The target *hsp* genes showed different expression patterns in response to heat shock. We found that *hsf1* expression was not upregulated (Fig. 5A), nor did it differ between the two species. In contrast, *hsp83* and *hsc70-5* expression was upregulated in both species (*C. bombycina*: $F_{6,14}$=6.64, P<0.002; *C. mauritanica*: $F_{6,14}$=31.15, P<0.0001; Tukey *post hoc* tests: P<0.05 for both species; Fig. 5B,C) but no interspecific difference was found (two-tailed t-test, P>0.07 for both genes). In the case of *hsc70-4 h1*, gene expression was significantly upregulated in *C. mauritanica* (P<0.01) but not in *C. bombycina* (between-species difference: t=3.85, d.f.=4, P<0.02; Fig. 5D). Finally, *hsc70-4 h2* expression was upregulated in both species (P<0.01), and the species differed in their expression patterns (t=3.67, d.f.=4, P<0.03; Fig. 5E).

DISCUSSION

Compared with related mesophilic species, thermophilic species are expected to have higher constitutive expression but lower induced expression of HSP-related genes (Evgen'ev et al., 2014). The results of our study support this prediction: in the highly thermophilic *C. bombycina*, constitutive expression of HSC70 was higher than in *C. mauritanica*, but induced expression of *hsc70-4 h1* and *hsc70-4*

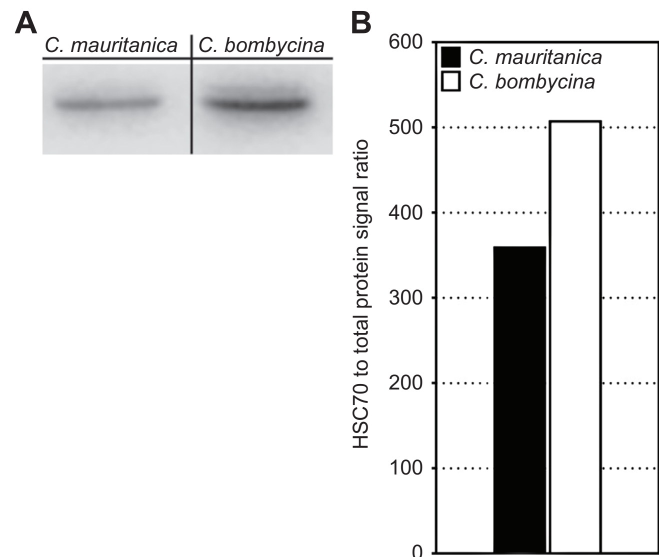

Fig. 4. Western blotting of HSC70 proteins from *C. mauritanica* and *C. bombycina* workers. Total protein extracts were separated and the presence of HSC70 was assessed using monoclonal antibodies. (A) Moderate levels of HSC70 were detected in *C. mauritanica* while higher levels of HSC70 were detected in *C. bombycina*. (B) HSC70 normalised to total protein signal ratio. Results are representative of three replicates.

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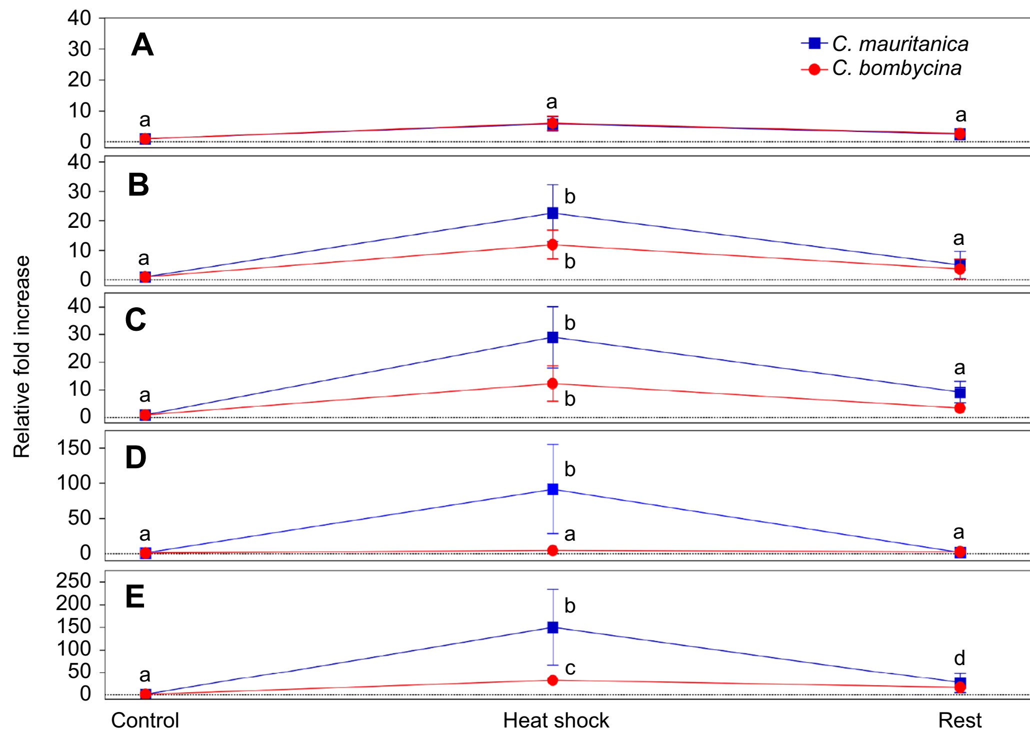

Fig. 5. Relative fold increase in expression of six genes in response to a heat shock in *C. mauritanica* and *C. bombycina* across different treatments. Total RNA was extracted from three groups of six individuals each in control conditions (25°C for 3 h), exposed to heat shock (40°C for 3 h) or subsequently rested (25°C for 1 h). Expression of (A) *hsf1*, (B) *hsc70-5*, (C) *hsp83*, (D) *hsc70-4 h1* and (E) *hsc70-4 h2* was normalised to that of *β-actin* and *eEf-1* (see Materials and methods). Mean±s.d. fold change is presented. Different lowercase letters indicate a significant difference between treatments (one-way ANOVA and Tukey *post hoc* test, $P<0.05$).

h2 in response to heat shock was lower. Furthermore, *C. bombycina* workers suffered less protein damage upon heat shock and exhibited lower rates of heat hardening. Although the strength of inference that can be drawn from comparison of two species may be limited (Garland and Adolph, 1994), our study strongly supports that the HSR is highly correlated to the species' thermal environment.

Heat stress and gene upregulation

Few studies have investigated HSP synthesis under heat-stress conditions in ants. For those that have, HSP70 synthesis has largely been the focus (Gehring and Wehner, 1995; Maisov et al., 2007). However, Nguyen et al. (2016) recently showed that the primary inducible gene involved in HSR in *Drosophila* – *hsp70* – has been completely lost in Hymenoptera, which instead employs two *hsc70-4* paralogues. Like Gehring and Wehner (1995), we found that HSC70 is constitutively expressed in *C. bombycina*. We also found comparatively moderate levels of HSC70 in *C. mauritanica* under control conditions. High constitutive expression of molecular chaperones has been reported in a number of ectotherms living under stressful conditions (e.g. elevated temperature, high salinity), including molluscs (Dong et al., 2008), echinoderms (González et al., 2016), dipterans (Rinehart et al., 2006; Garbuz et al., 2008) and lizards (Zatsepina et al., 2000). The sustained production of HSC70 by *C. bombycina* and *C. mauritanica* is probably an adaptation for coping with heat. We expect elevated constitutive expression of HSC70 to occur across the entire *Cataglyphis* genus, as all its members are thermophilic.

To the best of our knowledge, only three studies have explored the expression of HSP-related genes in response to heat stress in ants. Beside the elevated constitutive level of HSC70 in *C. bombycina*, Gehring and Wehner (1995) also showed that HSC70 synthesis carries on at temperatures as high as 45°C in this species. In contrast, this synthesis is abolished above 40°C in the wood ant *Formica polyctena*, a mesic species closely related to *Cataglyphis* that inhabits the northern part of the Paleartic region. The second study investigated *hsp60*, *hsp75* and *hsp90* expression in response to temperature in the wood ant *Formica cinerea* and found no significant effects (Ślipiński et al., 2015). The third study compared heat-shock-related expression patterns of several *hsp* genes between *Pogonomyrmex barbatus*, an ant living in hot and arid climates, and *Aphaenogaster picea*, an ant living in cool woodlands (Nguyen et al., 2016). These authors showed that expression of *hsc70-4*, *hsp83* and *hsp40* was upregulated in response to heat stress and the species differed significantly in relative expression levels. More specifically, upregulation occurred at higher temperatures in *P. barbatus*. In contrast, induced expression of *hsc70-5*, *hsc70-3* and *hsp60* was unaffected by heat stress. Our findings add to the knowledge about HSR in ants. They show that, in *Cataglyphis*, *hsf1* expression is not induced by heat shock. This result suggests that ambient concentrations of cytosolic HSF1 are sufficient to induce the expression of *hsp* genes under conditions of heat stress. Consistent with this hypothesis, in previous studies, differences in HSF1 activation and binding to HSEs, as opposed to upregulation, are crucial to the constitutive and induced expression of HSPs (Zatsepina et al., 2000). In response to heat shock, *hsp83* expression was moderately upregulated in both *C. bombycina* and *C. mauritanica*. Upregulation of the *hsp90* gene family in response to heat stress is well documented in ectotherms (Rinehart et al., 2007; Choi et al., 2014; González et al., 2016; Nguyen et al., 2016) and, based on our findings, also appears to be conserved in *Cataglyphis*. We also observed an increase in *hsc70-5* expression in response to heat shock. This result was somewhat unexpected as this cognate has

not been reported to demonstrate heat-inducible expression in the other ant species studied to date (Ślipiński et al., 2015; Nguyen et al., 2016). The search for conserved HSEs in the promoter region of *hsc70-5* in *Cataglyphis*, as well as in other Formicinae, might yield further insights into whether this cognate is a unique tool used by *Cataglyphis* ants to increase proteome stability.

We found that, among all the *hsp* genes examined, *hsc70-4* showed the greatest variation in expression in response to heat shock. This pattern was also observed in *P. barbatus* and *A. picea* (Nguyen et al., 2016). We also discovered differences in induced expression between species. First, while expression of both *hsc70-4* paralogues was upregulated in *C. mauritanica*, expression of only one (*hsc70-4 h2*) was upregulated in *C. bombycina*. Consequently, in the latter species, *hsc70-4 h1* might have evolved a housekeeping function to enhance proteome stability. This difference was not surprising, as the *hsc70-4* family shows member duplication in various insects, suggesting a wide array of functional shifts have taken place over the course of evolution. In the mosquito *Culex quinquefasciatus*, differences in paralogue inducibility were inferred from the absence of HSEs in the promoter region of one copy of the gene, which is consistent with a housekeeping function; the other copy might remain heat inducible (Nguyen et al., 2016). Our data suggest the scenario may be similar in *C. bombycina*. Although expression of *hsc70-4 h2* was induced at high levels in both species, the relative increase was higher in *C. mauritanica.* The relatively lower levels of induced expression in *C. bombycina* were expected and make sense given the high constitutive HSC70 concentrations observed. Indeed, a trade-off between the constitutive and induced expression of HSP70 has been documented in a number of organisms living under stressful conditions (Rinehart et al., 2006; Dong et al., 2008; Clark and Peck, 2009; Zatsepina et al., 2016). This trade-off seems at least partially conserved in *Cataglyphis*: compared with the more mesophilic *C. mauritanica*, the more thermophilic *C. bombycina* had relatively higher constitutive expression of HSC70 but relatively lower induced expression of the target *hsp* genes. This shift towards higher constitutive expression in *C. bombycina* may have been selected for by the narrow range of extremely high temperatures that workers experience upon leaving the nest to forage in the Sahara desert. In *C. mauritanica*, the shift towards induced expression might allow workers to cope with the less intense but more variable temperatures of the Atlas Mountains. Overall, these results suggest key differences exist in *Cataglyphis* HSRs, probably as a result of the contrasting environments the species inhabit.

Protein damage

Proteotoxic stressors such as heat denature proteins, causing them to aggregate because their insoluble residues become exposed (Chen et al., 2002). As predicted, levels of protein aggregation were significantly higher in heat-shocked *C. mauritanica* workers than in heat-shocked *C. bombycina* workers. This result is consistent with our western blot analyses, which show that constitutive expression of HSC70 is greater in the more thermophilic *C. bombycina* than in *C. mauritanica*. A similar positive correlation between levels of constitutively expressed molecular chaperones and proteome stability has been previously documented in the Antarctic midge, *Belgicana antartica*, which also tolerates extreme environmental conditions (Rinehart et al., 2006).

Heat hardening and thermotolerance

As predicted, in the heat-hardening experiment, the survival rate of *C. mauritanica* workers was significantly higher, strongly suggesting the species uses heat hardening to induce thermotolerance. This finding is consistent with our gene expression results, which showed that *C. mauritanica* workers strongly upregulated the expression of the two *hsc70-4* cognates in response to heat stress. In contrast, in *C. bombycina*, only *hsc70-4 h2* expression was upregulated, and to a lesser extent than in *C. mauritanica*. As the upregulation of *hsp70* expression is closely tied to survival and heat hardening (Feder and Hofmann, 1999; Bahrndorff et al., 2009), our results suggest that *C. mauritanica* can acquire a high level of induced thermotolerance while *C. bombycina* cannot. In the latter species, a 2 h pre-treatment at 37°C increased mortality of workers subsequently exposed to a severe heat stress (3 h at 45°C). It has been shown that pre-exposure to temperatures above the range inducing thermotolerance can cause thermosensitivity instead (Yocum and Denlinger, 1993). Exposure to such critical temperatures results in a rather quick mortality among individuals and limits protein synthesis to molecular chaperones (Yocum and Denlinger, 1993). Contrary to these expectations associated with thermosensitivity, we did not observe any significant mortality of *C. bombycina* workers after 3 h exposure to 37 or 40°C. Furthermore, we detected ongoing transcriptional activity of non-molecular chaperone-related genes such as housekeeping genes (*β-actin*, *eEF-1*) and *hsf1* (Fig. 5A) after 3 h at 40°C (see Results, 'Heat stress and gene upregulation'). Therefore, and regardless of the highly thermophilic nature of *C. bombycina* workers whose CT_{max} reaches 53.6°C (Hoffmann et al., 2013), it is unlikely that exposure to 37°C is stressful enough to induce thermosensitivity or impair the HSR. Rather, the lower survival rate of *C. bombycina* workers after pre-treatment probably stems from the combination of prolonged exposure to above-optimal temperatures and lessened HSC70 up-regulation.

Another unexpected result of this study is that the two species suffered equal mortality rates (approximately 60%) when exposed to severe heat stress (Fig. 2). Given that *C. bombycina* workers constitutively express more HSC70, one would predict that this species should be able to tolerate higher body temperatures. One tentative explanation may involve a combination of worker morphology and experimental design. In *C. bombycina*, the high constitutive levels of HSC70 could adaptively complement cuticle morphology in generating resistance to the heat stress induced by sunlight under natural conditions. Indeed, recent studies have shown that the dorsal side of *C. bombycina* workers (but not of *C. mauritanica* workers) is covered by a unique array of prism-shaped hairs that reflect incident sunlight and are responsible for the species' silver colour (Fig. 1) (Shi et al., 2015; Willot et al., 2016). The hairs are thermally protective in the visible and near-infrared range of the electromagnetic spectrum, reducing the absorption of sunlight energy and leading to lower internal body temperatures in workers. Sensitive tissues (e.g. central neural tissue) that are protected by such hairs might not need as high a level of constitutive molecular chaperones as ventral organs, which are directly exposed to infrared radiation emanating from the hot ground. Therefore, we cannot exclude the possibility that the high levels of constitutive HSC70 detected in whole-body extracts from *C. bombycina* stem from the localised accumulation of HSC70 in specific tissues. Moreover, hairs have elevated transmissivity in the mid-infrared range, allowing radiation to bypass the ant's protective coat more readily at those wavelengths (Shi et al., 2015). As a consequence, we may have observed similar mortality rates in the two species because our experimental design did not reproduce the natural balance of incoming solar energy. Instead, homogeneous infrared radiation came from all directions, bypassing *C. bombycina*'s protective coat and hitting critical organs. A tissue-specific

comparison of HSC70 constitutive expression in *C. bombycina* might reveal key differences in heat tolerance strategies.

Finally, in all our experiments the ants were exposed to a 3–5 h heat stress. This corresponds to somewhat artificial conditions, as *C. bombycina* foragers frequently use thermal refuges in order to dump excessive internal heat in the wild (Wehner et al., 1992). The length of exposure to heat stresses in our experiments was chosen to take into account the full extent of the HSR and compare results with existing data (Gehring and Wehner, 1995). We showed that in both species of *Cataglyphis* studied, mRNA of up-regulated genes following heat shock abruptly decreased after 1 h respite time (Fig. 5B–E). We are therefore confident that the lessened *hsc70* mRNA induction observed in *C. bombycina* does not result from an experiment in which 4 h at 40°C would allow significant mRNA decay.

Conclusions

In summary, this study demonstrates that the more thermophilic ant species, *C. bombycina*, has higher constitutive expression of HSC70 and lower induced expression of *hsp* genes in response to heat stress than the more mesophilic ant species *C. mauritanica*. Consequently, *C. bombycina* workers show increased protein stability when exposed to heat stress but are less prone to acquiring induced thermotolerance by means of heat hardening. These results confirm that HSR patterns are highly representative of the species' thermal environment. More generally, our findings provide insights into the physiological plasticity needed to cope with fluctuating or novel temperature regimes. For example, global warming might prove to be an even greater challenge for species with less-plastic HSP systems than for species that are better equipped to cope with fluctuating body temperatures.

Acknowledgements
We thank E. Goormaghtigh and G. Vandenbussche for their advice concerning the protein aggregation experiments and the use of their laboratory. We are grateful to R. Soin and A. Nazih for their help with the RTq-PCR analyses and the western blots. Thanks to J. Pearce for her language editing services.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Q.W., C.G. and S.A. conceived and planned the study. Q.W. and S.A. collected samples. Q.W. and C.G. performed molecular work and analysed the data. All authors contributed to drafting the article, approved the final published version and agree to be held accountable for all aspects of the work.

Funding
This work was supported by a Fonds pour la Formation à la Recherche dans l'Industrie et dans l'Agriculture (FRIA) scholarship (to Q.W.) and CDR funding (to S.A.; J.0151.16) from the Belgian Fund for Scientific Research (Fonds de la Recherche Scientifique, FRS-FNRS).

Supplementary information
Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.154161.supplemental

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Table S1. Primer sets for qPCR including housekeeping genes.

Gene	Primer 5'-3'	Amplicon Length (bps)
β-actin (forward)	TATTATTGCTACACCTTTCCCTAAG	
β-actin (reverse)	TTCGACTAACAGATCCAACTAAAC	76
Ef1-β (forward)	AGAAAGCCCAGAAGAAGAAATAAC	
Ef1-β (reverse)	CACCCATATTCGCACGGA	76
hsc70-4_1 (forward)	CTCCGCTCTCTCTGGGTATC	
hsc70-4_1 (reverse)	CGGGATGGTAGTGTTCCTCTT	74
hsc70-4_2 (forward)	ACCGACTAGAACTATACTGTGAAC	
hsc70-4_2 (reverse)	TTGAACCCGTCGAGAAAGC	74
hsc70-5 (forward)	ACATGGTTGAATAGTACGCTTAA	
hsc70-5 (reverse)	TGAATCTTAAACTGTCCAGAAGTAA	74
hsp83 (forward)	TGACGAAATGTGCTCTCTTAAAG	
hsp83 (reverse)	AGTGATGTAGTAAATGTGCTTCTG	73
hsf1 (forward)	TGTAGTATGTATTGTAACTCTCAGTGA	
hsf1 (reverse)	CTCCATTCTCCAACATCCTAGATT	80