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RESEARCH ARTICLE

Mitochondrial metabolism and oxidative stress in the tropical cockroach under fluctuating thermal regimes

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ABSTRACT

The cockroach *Gromphadorhina coquereliana* can survive at low temperatures under extensive periods of cold stress. To assess energy management and insect adaptation in response to cold, we measured mitochondrial activity and oxidative stress in muscle and fat body tissues from *G. coquereliana* under a fluctuating thermal regime (FTR; stressed at 4°C for 3 h on 3 consecutive days, with or without 24 h recovery). Compared with our earlier work showing that a single exposure to cold significantly affects mitochondrial parameters, here, repeated exposure to cold triggered an acclimatory response, resulting in unchanged mitochondrial bioenergetics. Immediately after cold exposure, we observed an increase in the overall pool of ATP and a decrease in typical antioxidant enzyme activity. We also observed decreased activity of uncoupling protein 4 in muscle mitochondria. After 24 h of recovery, we observed an increase in expression of antioxidant enzymes in muscles and the fat body and a significant increase in the expression of UCP4 and HSP70 in the latter. This indicates that processes related to energy conversion and disturbance under cold stress may trigger different protective mechanisms in these tissues, and that these mechanisms must be activated to restore insect homeostasis. The mitochondrial parameters and enzymatic assays suggest that mitochondria are not affected during FTR but oxidative stress markers are decreased, and a 24 h recovery period allows for the restoration of redox and energy homeostasis, especially in the fat body. This confirms the crucial role of the fat body in intermediary metabolism and energy management in insects and in the response to repeated thermal stress.

KEY WORDS: UCP, HSP, Bioenergetics, ROS, Insect, *Gromphadorhina coquereliana*

INTRODUCTION

Because temperate and polar species exhibit obvious adaptations to cold temperatures, their responses to cold stress have been studied in detail (Chen et al., 1987; Clark et al., 2009; Clark and Worland, 2008; Czajka and Lee, 1990; Montiel et al., 1998; Teets et al., 2012). Nevertheless, insects that originate in tropical or semitropical regions are not necessarily completely deficient in adaptative mechanisms or characteristics that allow them to resist cold stress. Indeed, some tropical species may periodically be exposed to low temperatures (e.g. at high altitudes) and therefore have developed strategies to cope with such conditions (Gibert et al., 2001; Goto and Kimura, 1998). For instance, some cockroaches, which are mostly of tropical origin, possess mechanisms that allow them to survive cold stress (Chowański et al., 2017, 2015; Lubawy et al., 2019). This is true of *Gromphadorhina coquereliana*, a cockroach that is endemic to the tropical island of Madagascar and is capable of surviving cold and freezing stress (Chowański et al., 2017, 2015; Lubawy et al., 2019; Slocinska et al., 2017).

Tolerance to cold stress results from various adaptations; among them, adaptations of the mitochondria are increasingly recognized as playing a key role (Chatterjee et al., 2022 preprint; Havird et al., 2020; Lubawy et al., 2022; Menail et al., 2022). During cold stress, mitochondria in insects function as cellular hubs that link metabolism, stress sensing, signalling and cell survival (Bohovych and Khalimonchuk, 2016; Breda et al., 2019; Lane, 2018; Monlun et al., 2017; Vakifahmetoglu-Norberg et al., 2017) and are also targets of cold-stress-associated damage (Lubawy et al., 2022). To date, few studies have been conducted on the cellular bioenergetics of insects after exposure to low temperatures and the results often vary with experimental context and species (Chowański et al., 2017; Colinet et al., 2017; Da-Ré et al., 2014). Exposure to low temperature was shown to alter ATP levels and mitochondria-driven energetic homeostasis (Colinet, 2011; Pullin and Bale, 1988); these changes may then, in turn, lead to oxidative stress if the production of reactive oxygen species (ROS) overcomes the antioxidative response capabilities of the mitochondria (Lubawy et al., 2022; Monaghan et al., 2009). Cold stress may decrease ATP production during aerobic metabolism, thus limiting the active transport of ions below passive flux (Dollo et al., 2010; Overgaard and MacMillan, 2017). Cold-induced promotion of ATP synthesis has been reported in several studies (Colinet, 2011; Macmillan et al., 2012). Hence, insects in which injury and death are related to chilling (chill-susceptible and chill-tolerant insects) should maintain their mitochondrial function in order to preserve ATP production. Cold stress also affects mitochondrial and nuclear gene expression patterns, including the expression of genes that encode mitochondrial energetic machinery proteins or heat shock proteins (HSPs), which act to protect cells and enable them to cope with the effects of stress (Ballard et al., 2007; Camus et al., 2017; Colinet, 2011; Colinet et al., 2018; Da-Ré et al., 2014; Dollo et al., 2010; El-Shesheny et al., 2016; Joanisse and Storey, 1994; Macmillan et al., 2012; Pullin et al., 1990; Ramirez et al., 2021; Singh et al., 2013; Teets et al., 2020). It was suggested that mitochondria can also play a role in heat dissipation by uncoupling respiration to protect the cell from the detrimental effects of cold stress (Da-Ré et al., 2014; Ulgherait et al., 2020). Furthermore, the role of mitochondria under cold stress conditions may vary depending on the species, the climatic zone in which they naturally occur and whether they are acclimated to cold stress (for details, see Lubawy et al. (2022) and references within). Depending on their survival strategy, insects fall

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into one of three categories: (i) insects that are capable of maintaining the active transport of ions during cold stress, for which sufficient ATP production is needed (and the ability to maintain ATP production most likely affects cold tolerance); (ii) diapausing insects, in which metabolic depression is observed; and (iii) insects capable of surviving when their internal fluids are frozen, i.e. 'the extreme survivors' (for details, see Lubawy et al., 2022 and references therein).

Understanding mitochondrial bioenergetic parameters under cold stress conditions is increasingly considered to be an important issue (Chowański et al., 2017; Colinet et al., 2017; Havird et al., 2020; Menail et al., 2022; Štětina et al., 2020). However, studies on the bioenergetics of insects have often been limited because of technical constraints, resulting in a lack of knowledge about the mitochondrial functions and adaptations in changing thermal environments (Menail et al., 2022). To date, only a few studies have investigated mitochondrial activity in cold-stressed and/or acclimated insects (Chowański et al., 2017; Colinet et al., 2017; Da-Ré et al., 2014; Havird et al., 2020). In *G. coquereliana*, a single exposure to cold causes significant changes in mitochondrial respiration activity. After 3 h at 4°C, the oxygen consumption of isolated mitochondria in the resting (state 4) and phosphorylating (state 3) states was altered. Both in muscle and fat body tissues, an increase in state 4 was observed, while state 3 was significantly decreased in the fat body. Moreover, cold stress induced changes in the activity of uncoupling protein 4 (UCP4) and the level of heat shock protein 70 (HSP70), causing significant increases in the HSP70 levels in both tissues (Chowański et al., 2017).

Taking the abovementioned factors into consideration, here, we determined how a fluctuating thermal regime (FTR), which is known to exert beneficial effects on insect thermal tolerance (Colinet et al., 2018), affects mitochondrial bioenergetics, the capacity to respire upon return to warm conditions and whether it evokes oxidative stress in this tropical insect. To determine how the *G. coquereliana* cockroach manages energy sources to adapt to low temperatures, we partially mimicked the conditions that occur in the natural environment. The lowest recorded temperature in Antananarivo over the last 40 years was 2.2°C, and over this time, the temperature dropped below 5°C at night for 3–4 h approximately 100 times (http://meteo-climat-bzh.dyndns.org/index.php?page=stati&id=769). Hence, we designed our experiments to simulate a few very cold nights during the winter. We repeatedly exposed *G. coquereliana* cockroaches to cold (3 h of cold stress for three consecutive days) and collected samples of the fat body, which is an insect tissue that is analogous to mammalian liver and adipose tissue, and leg muscle tissue (highly metabolic and energy-dependent tissue) (Slocinska et al., 2011) immediately after the last exposure and after 24 h of recovery (Fig. S1).

We assumed that the FTR would trigger adaptive mechanisms that result in undisrupted mitochondrial activity and capacity to respire (compared to a single exposure to cold) (Chowański et al., 2017, 2015) and an increase in oxidative stress followed by the induction of protective, antioxidant mechanisms. We hypothesized that during the recovery time (24 h), these protective mechanisms would be activated to restore the appropriate energetic and redox homeostasis in the insect. Hence, we determined the bioenergetic parameters of mitochondria that were isolated from these tissues (phosphorylating state of respiration and coupling of the mitochondria by determination of respiratory control ratio), as well as the level of oxidative stress. We measured the protein level of HSP70, which is considered the major inducible chaperone protein that is involved in the cold stress response (Colinet et al., 2010b; Dumas et al., 2019; Yi et al., 2018). Additionally, we measured the activity of UCP4, which has been implicated in the reduction of free radicals (Alves-Bezerra et al., 2014; Slocinska et al., 2016) or in the generation of heat (Da-Ré et al., 2014; Ulgherait et al., 2020), as well as the activities of major antioxidant enzymes, including superoxide dismutase (SOD) and catalase (CAT). Finally, we measured the total pool of ATP, which is the principal molecule for storing and transferring energy (Colinet, 2011).

MATERIALS AND METHODS

Insect rearing and tissue isolation

Cockroaches [*Gromphadorhina coquereliana* (Saussure 1863)] were reared in continuous colonies under laboratory conditions of 28°C, approximately 65% relative humidity, and a 12 h light:12 h dark cycle in the Department of Animal Physiology and Development, AMU in Poznań, Poland. Food (lettuce, carrots, and powdered milk) and water were provided *ad libitum* as previously described (Lubawy et al., 2019). Only adult males approximately 6 cm in length and 5.5 g in weight were used in the experiments. For all experiments, cockroaches were anaesthetized as previously described (Chowański et al., 2015); then, the insects were decapitated and the legs were removed. The legs were washed with physiological saline (PS: 139 mmol l^{-1} NaCl, 5 mmol l^{-1} KCl, 4 mmol l^{-1} $CaCl_2$) to remove the rest of the haemolymph. For muscle isolation, only the femur was used. The fat body was isolated from the abdomen using microsurgical forceps and then washed with PS. Malpighian tubules and tracheas were removed with microsurgical tweezers under a stereoscopic microscope. Tissue isolation was performed on ice. All basic reagents were purchased from Merck (Merck, Warsaw, Poland) unless stated otherwise.

Thermal treatments

Adults were subjected to low temperature stress by placing them (3–5 individuals together) in plastic boxes (14×8×12 cm) with food, and then the boxes were placed in an incubation chamber (MIR-154, PHCBI, The Netherlands) at 4°C and approximately 65% humidity. In the wild, this species can occasionally be exposed to temperatures of approximately 3–6°C for a few hours at night and the average annual temperatures reach 28°C (Chowański et al., 2015), we devised a thermal regime that mimicked these conditions. The insects were exposed to cold at 4°C for 3 h per day for 3 consecutive days, and then the insects were sampled for experiments immediately after the last cold exposure (sampling time 1, denoted ST1) or at 24 h to allow them to fully recover (denoted sampling time 2, ST2) (Fig. S1). The control animals were maintained under the same temperature and light conditions that were used in the primary culture (28°C, 65% RH, 12 h light:12 h dark). The samples for all experiments were collected immediately after the end of the thermal treatments.

Mitochondria isolation

Muscle mitochondria-enriched fraction was obtained using previously described methods (Chowański et al., 2017). Isolation was performed in medium containing 100 mmol l^{-1} KCl, 50 mmol l^{-1} Tris-HCl (pH 7.4), 1 mmol l^{-1} K_2HPO_4 and 0.2% bovine serum albumin (BSA). Muscle tissue was dissected into small pieces within a minute and then homogenized in a glass Teflon homogenizer. Tissues from 3–5 individuals were pooled to generate a single sample. The homogenate was then centrifuged for 10 min at 500 ***g***. The acquired supernatant was centrifuged for 10 min at 10,000 ***g***. The pellet, which contained the mitochondria, were centrifuged at 6000 ***g*** for 10 min. The final mitochondrial

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pellet was resuspended in medium containing 0.2 mol l^{-1} mannitol, 0.1 mol l^{-1} sucrose, 10 mmol l^{-1} Tris-HCl (pH 7.4) and 0.1 mmol l^{-1} EDTA and stored on ice for subsequent assays.

The mitochondria were isolated from the fat body in medium containing 0.25 mol l^{-1} sucrose, 1 mmol l^{-1} EDTA, 5 mmol l^{-1} Tris-HCl and 1% BSA. The tissue was homogenized in a glass Teflon homogenizer. The homogenate was then centrifuged for 10 min at 800 ***g***, and the collected supernatant was centrifuged at 12,000 ***g*** for 10 min. The acquired pellet was then washed twice in a medium containing 0.25 mol l^{-1} sucrose, 1 mmol l^{-1} EDTA, and 5 mmol l^{-1} Tris-HCl and was centrifuged at 8000 ***g*** for 10 min. The final pellet that contained the mitochondria was resuspended in the same medium.

All these procedures were performed at 4°C. Mitochondrial protein concentrations were determined using a Direct Detect® Infrared Spectrometer (Merck Millipore).

Measurement of oxygen consumption by mitochondria

Oxygen consumption of mitochondria that were isolated from *G. coquereliana* muscle and fat body was measured at room temperature with a Clark-type electrode (Oxytherm, Hansatech) in 0.8 ml incubation medium containing 0.2 mol l^{-1} mannitol, 75 mmol l^{-1} sucrose, 10 mmol l^{-1} KCl, 0.1 mmol l^{-1} EDTA, 10 mmol l^{-1} K_2HPO_4 and 10 mmol l^{-1} Tris-HCl (pH 7.4). Succinate (10 mmol l^{-1}) or pyruvate (10 mmol l^{-1}) plus malate (10 mmol l^{-1}) were used as oxidizable substrates to measure fat body and muscle mitochondrial respiration, respectively. Substrates were delivered to incubation medium after the addition of mitochondria (0.4 mg) and then ADP was added. The respiratory control ratio (RCR) was calculated as the ratio of state 3 respiration (phosphorylating state after addition of ADP) to state 4 (resting state before ADP). The total amount of oxygen that was consumed during the phosphorylating respiration that was induced by a pulse of ADP (250 nmol) was used to calculate the ADP/O ratio. O_2 uptake values are presented in nmol O_2 min^{-1} mg^{-1} protein.

To induce uncoupling protein 4 (UCP4) activity-mediated respiration, measurements were performed in the presence of palmitic acid (15 µmol l^{-1}). Palmitic acid-induced UCP4 activity was inhibited by the addition of 2 mmol l^{-1} GTP. To exclude the activities of the ATP/ADP antiporter and ATP synthase, carboxyatractyloside (1.5 µmol l^{-1}) and oligomycin (1 mg per 1 mg of mitochondrial protein) were added to the incubation medium.

Measurement of cytochrome *c* oxidase (COX) activity after cold stress

The maximal COX activity was assessed as previously described (Koziel et al., 2012) with 0.25 mg mitochondrial protein without the exogenous addition of respiratory substrate and in the presence of antimycin A (10 µmol l^{-1}), 8 mmol l^{-1} ascorbate, 0.06% cytochrome *c* and up to 2 mmol l^{-1} *N*,*N*,*N′N′*-tetramethyl-p-phenylenediamine (TMPD), which were sequentially added. The maximal O_2 consumption by COX (complex IV) was determined by measuring the rate of its consumption following the addition of TMPD. The experiments were performed with four biological replicates per thermal treatment and control; each replicate consisted of mitochondria that were isolated from five pooled individuals.

Superoxide level

The level of O_2^- released from mitochondria was determined by measuring the rate of nitroblue tetrazolium (NBT) reduction by isolated mitochondria. NBT reduction was measured in 1.2 ml of incubation medium in the presence of mitochondria (0.6 mg), 10 mmol l^{-1} succinate (fat body) or 10 mmol l^{-1} malate and 10 mmol l^{-1} pyruvate (muscle), which were added after the addition of NBT at a final concentration of 0.07 mg ml^{-1}. The reduction was measured at 560 nm (UV 1602, Shimadzu).

Isolation of mRNA and generation of cDNA

Isolated tissues (fat body or muscles) were transferred to 300 µl RNA lysis buffer (Zymo Research, Irvine, CA, USA) and homogenized for 2 min using a pellet homogenizer (Kimble Chase, USA). For each treatment and control, at least three biological replicates were used, each consisting of tissues that were collected from three pooled individuals. The homogenized tissues were immediately frozen in liquid nitrogen and then stored at −80°C. Total RNA was extracted using the Insect RNA MicroPrep™ Kit (Zymo Research Corp., Irvine, CA, USA) according to the manufacturer's protocols. The protocol included in-column DNase I treatment to remove traces of gDNA and prevent contamination of the RNA samples. ReverAid™ Reverse Transcriptase (Fermentas, Waltham, MA, USA) was used to generate cDNA according to the manufacturer's protocols. Quantitative real-time PCR (RT-qPCR) was performed on a Corbett Research RG-6000 Real Time PCR Thermocycler (QIAGEN, Venlo, The Netherlands) with a Sensitive RT HSPCR Mix SYBR® kit (A&A Biotechnology s.c, Gdańsk, Poland). In addition to biological replicates, at least two technical replicates were performed. The expression level of the gene encoding *G. coquereliana* 18S rRNA was used as a control. To check for potential foreign contamination of samples, 'no template' negative controls (DNA/RNA-free water) were tested. The relative expression was calculated using the $2^{\Delta\Delta}$Ct method. The primers used for specific genes were as follows; *18S rRNA* forward primer: CAGGGTTCGATTCCGGAGAG; *18S rRNA* reverse primer: TGCTCATTCCGATTACGGGG; *UCP4* forward primer: CCGAA-CATGCAAAGAGCAGC; *UCP4* reverse primer: AGCCACTAGTC-CAGCTACCA; *SOD* forward primer: TGTCCGGTCTAG-GAAAAGGC; *SOD* reverse primer: GGGTTGAAATGAGCAC-CAGC; *CAT* forward primer: TTCAGTTACGCCGACACACA; *CAT* reverse primer: CCACTTCTCCTGATGGTGGG; *HSP70* forward primer: AGACACTCATCTTGGCGGTG; *HSP70* reverse primer: ACAGTGTTCGCTTAGCCCTC.

Determination of protein content in fat body and muscle tissue

After tissue collection, the samples were homogenized in physiological saline and centrifuged at 10,000 ***g*** for 10 min. The supernatant was used to determine the protein content in the soluble fraction. The measurement was conducted with a Direct Detect Infrared Spectrometer (Merck Millipore, Warsaw, Poland). A total of 2 µl supernatant was placed on the PTFE membrane and left to dry for 3 min. The protein content was then measured. Bovine serum albumin (Merck Millipore, Warsaw, Poland) was used as a standard. Samples were snap frozen in liquid nitrogen and stored at −80°C for further analyses.

Immunodetection

The soluble protein fraction was resuspended in sample buffer. SDS-PAGE (using a 14% polyacrylamide resolving gel) using 40 µg protein per well followed by western blotting was performed as previously described (Chowański et al., 2015). Rabbit polyclonal primary antibodies against Heat shock protein 70 (HSP 70, AS09592 from Agrisera, Vännäs, Sweden), Catalase (CAT, ab16731 from Abcam, Cambridge, UK) and Superoxide dismutase 1 (SOD, ab13498 from Abcam, Cambridge, UK) were used at 1:500 dilution, 1:300 dilution and 1:250 dilution,

respectively. The blots that were used for the immunodetection of HSP70 and SOD were incubated with primary antibodies for 90 min, whereas those that were used for the immunodetection of CAT were incubated with antibodies overnight. Goat anti-rabbit secondary antibodies conjugated with horseradish peroxidase (Agrisera, AS09602) were used at dilutions of 1:10,000, 1:25,000 and 1:25,000, respectively, and incubated with the membranes for 1 h at room temperature. Antibodies against α-tubulin at a 1:500 dilution were used as a loading control. The protein bands were visualized using an Amersham ECL system and digitally quantified using ImageLab software (Bio-Rad, Warsaw, Poland).

ATP level determination

The total amount of ATP in analysed tissues was measured with a fluorescence assay (Molecular Probes ATP kit; A22066, Thermo Fisher Scientific, Warsaw, Poland) according to the method described previously by Tennessen et al. (2014) with some modification. The analysed tissues (muscles and fat body) were isolated directly after the last cold exposure (ST1) and after 24 h (ST2). The isolation was performed on ice, and the samples were placed in 1.5 ml Eppendorf tubes and kept on ice. Then, the samples were dried to a stable weight at 60°C under a vacuum (−0.9 atm) and weighed to determine the dry weight of the sample. In the next step, the samples were homogenized in 300 µl of 2.5% TCA solution and centrifuged for 5 min (4°C, 1400 rpm). The supernatant was transferred to new tubes, boiled for 5 min at 90°C and centrifuged at 12,000 rpm at 4°C for 15 min. The obtained supernatant was transferred to a new tube, frozen in liquid nitrogen and stored at −80°C until analysis. Prior to ATP level measurement, the ATP reaction mix was prepared according to the manufacturer's protocol. The samples were diluted with buffer (25 mmol l^{-1} Tris-HCl, 100 µmol l^{-1} EDTA, pH 7.8) 10- or 750-fold in the case of fat body and muscles, respectively. Next, 70 µl of sample was transferred to individual wells of a white, opaque 96-well plate and mixed with 70 µl ATP reaction mix containing luciferase. The luminescence was measured with a Synergy H1 Hybrid MultiMode microplate reader (BioTek, Winooski, Vermont, USA). The measurement was initiated 15 s after the reaction mix was added. The amount of ATP is present as picomoles of ATP per mg of dry tissue.

Lipid peroxidation

The lipid peroxidation level was determined by measuring thiobarbituric acid reactive substances (TBARS) using a TBARS assay kit (700870, Cayman Chemical, Tallinn, Estonia) according to the manufacturer's manual. Briefly, 100 µl of sample that was obtained as described above was mixed with 100 µl of 10% trichloroacetic acid (TCA) and 800 µl colour reagent (TBA, acetic acid, sodium hydroxide; Cayman Chemicals) in a 2 ml vial. Next, the vials were placed in vigorously boiling water. After 1 h, the vials were placed on ice and incubated for 10 min to stop the reaction. After centrifugation at 1600 ***g*** at 4°C for 10 min, the absorbance of the samples was measured at 530 nm using a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek, USA). The results were calculated from the standard curve of malonaldehyde (MDA) and are presented as µmol l^{-1} MDA $µg^{-1}$ protein.

Determination of antioxidant enzymatic activity

To determine the activity of superoxide dismutase (SOD) and catalase (CAT), we used a Superoxide Dismutase Assay Kit (706002, Cayman Chemical, Tallinn, Estonia) and Catalase Assay Kit (707002, Cayman Chemical, Tallinn, Estonia) according to the manufacturer's protocols, respectively. Both enzymes form a crucial part of the cellular antioxidant mechanism. Briefly, to measure SOD activity, 10 µl sample was combined with 200 µl radical detector [tetrazolium salt diluted in 50 mmol l^{-1} Tris-HCl, pH 8.0 containing 0.1 mmol l^{-1} diethylenetriaminepetaaceticacid (DPTA) and 0.1 mmol l^{-1} hypoxanthine] in a 96-well microplate. To initiate the reaction, 20 µl xanthine oxidase was added to each well and incubated for 30 min at room temperature on a shaker. After incubation, the absorbance of the samples was read at 540 nm using a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek, USA). The results were calculated from the standard curve of bovine erythrocyte SOD and are presented as the unit of SOD activity per ml per µg of protein (U ml^{-1} $µg^{-1}$). For the CAT activity, 20 µl of the samples were mixed with 100 µl of assay buffer (100 mmol l^{-1} potassium phosphate, pH 7.0) and 30 µl methanol in a 96-well plate. To initiate the reaction, 20 µl hydrogen peroxide was added and incubated for 20 min on a shaker. Then, 30 µl potassium hydroxide was added to terminate the reaction and 30 µl catalase purpald was added. After a 10 min incubation, catalase potassium periodate was added to each well and incubated for 5 min at room temperature. Absorbance of the samples was measured at 540 nm using a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek, USA). The results were calculated from the standard curve of formaldehyde and are presented as nmol min^{-1} ml^{-1} $µg^{-1}$ protein.

Statistical analysis

For statistical analysis, we used GraphPad Prism software v.9 (GraphPad Software, San Diego, CA, USA) (Department of Animal Physiology and Development AMU licence). Before statistical analysis, the normality of the distribution (the Shapiro–Wilk test) was evaluated. All data are presented as the means±s.e.m. of the indicated number of replicates (*n*). The statistical significance of differences between values of control insects and values of those exposed to thermal stress were determined using one-way ANOVA with Tukey's *post hoc* test (for data with normal distribution), Kruskal–Wallis test with Dunn's *post hoc* test (for data without normal distribution) or Student's *t*-test. Differences were considered statistically significant at: *$P≤0.05$, **$P≤0.01$ or ***$P≤0.001$.

RESULTS

The effect of cold on activity and coupling of mitochondria

We observed changes in mitochondrial efficiency during cold stress. In fat body tissue, after 3×3 h of cold stress, state 3 respiration decreased by 19.41%, which may indicate lower ADP-stimulated respiration and slightly lower ATP synthesis during cold stress. After recovery, this value increased significantly compared with immediately after the 3×3 h treatment (ST1) ($P=0.0463$) (Fig. 1A). In both tissues, no changes in state 4 respiration were observed among treatments (Figs 1B and 2B). Consequently, a decreasing trend in the RCR was observed after 3×3 h cold treatment. The RCR decreased by approximately 19.82% in the fat body and 19.95% in muscle, but neither of these changes was statistically significant (Figs 1C and 2C). Interestingly, at the same time, a trend of increasing ADP/O values was observed: these values increased by approximately 13.84% and 23.45% in the fat body and muscle, respectively (Figs 1D and 2D); however, this difference was not statistically significant. At 24 h after the last cold exposure, energy homeostasis was restored, and the bioenergetic parameters were similar to those observed in both tested tissues under control conditions.

The activity of UCP in muscle mitochondria was three times higher than that in fat body mitochondria (Fig. 3A,B). After repeated exposure to cold (3×3 h) and a recovery phase (3×3 h+24 h), the

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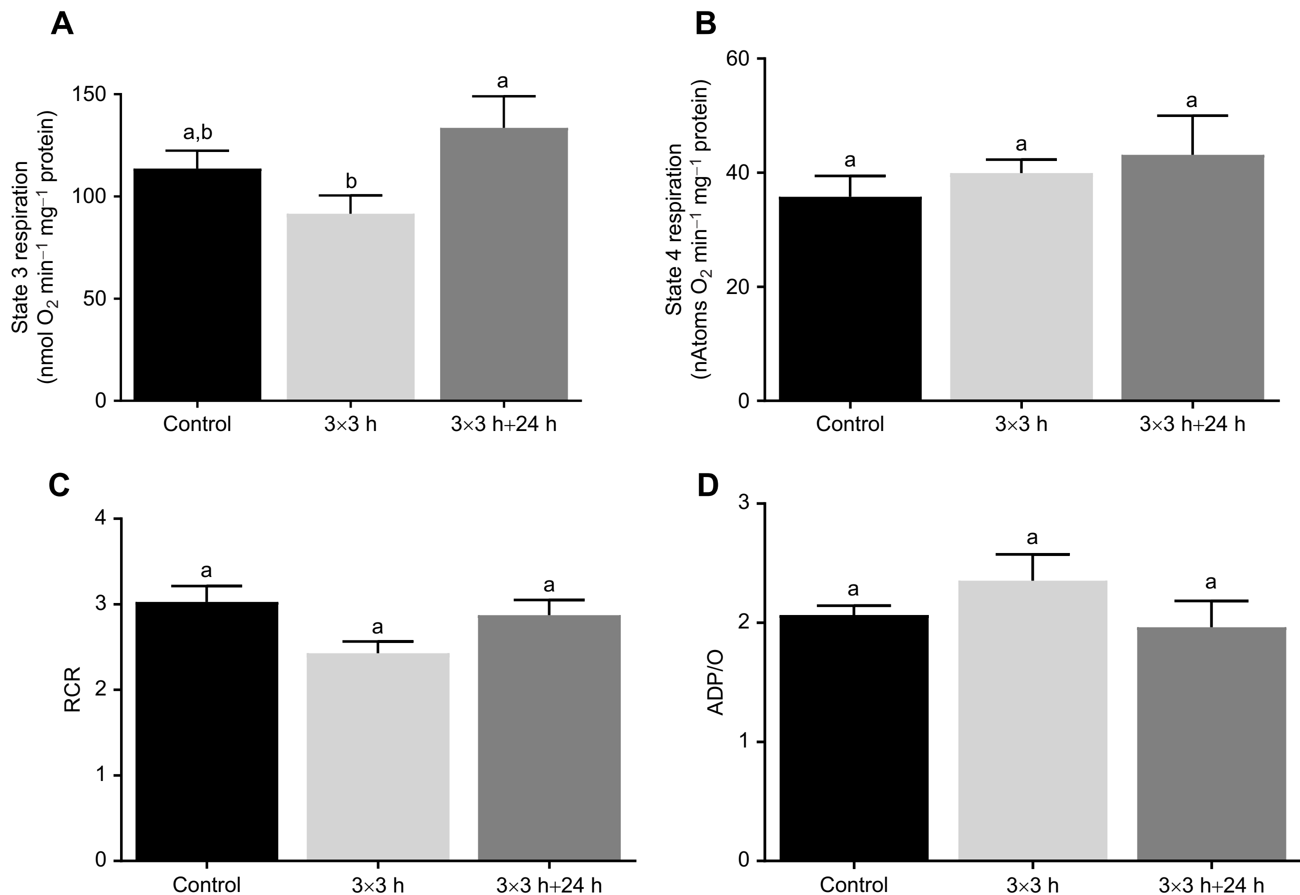

Fig. 1. Bioenergetic parameters of mitochondria isolated from the fat body tissue of the tropical cockroach *Gromphadorhina coquereliana*. (A,B) Respiration was measured in the presence of 10 mmol l^{-1} succinate and in the absence (state 4 respiration) or presence (state 3 respiration) of 400 µmol l^{-1} ADP. Changes in (C) the respiratory control ratio (RCR) and (D) ADP/O ratio of the fat body of *G. coquereliana* cockroaches following repeated exposure to cold (3×3 h) and after recovery (3×3 h+24 h). Data are means±s.e.m. (n=7). Different letters represent statistically significant differences between groups (P≤0.05). Statistical significance was determined using one-way ANOVA with Tukey's multiple comparisons test.

UCP activity in muscle was lower compared with the control, especially after the recovery (ST2) (Tukey's test: P=0.0395) (Fig. 3A). The level of *UCP4* in muscle exhibited a decreasing trend at the first timepoint (Tukey's test: P=0.1344) and an increasing trend at the second timepoint compared with the control (Tukey's test: P=0.0.8515) (Fig. 3C). However, no statistically significant changes were observed. In the fat body, UCP activity marginally increased in both variants, but the changes were not significant (P=0.7805; d.f.=2) (Fig. 3B). The expression level of *UCP4* varied significantly among the 3 conditions (P=0.0099; d.f.=2). Values were comparable to that of the control at the first timepoint, but it significantly increased (Tukey's test; P=0.0354) by 1.83-fold after recovery (Fig. 3D).

In the muscles, we observed a significant decrease in COX activity among the treatment groups (ANOVA P=0.0116; d.f.=2) (Fig. 4, left). Immediately after cold stress (3×3 h), the COX activity decreased by 22.02% (Tukey's test: P=0.0121) and a further decrease of 36.52% was observed after the recovery phase (Tukey's test: P=0.0094) compared with the control. The mitochondria isolated from the fat body exhibited significant changes in COX only immediately after cold stress (ST1), and this value was 41.80% higher than that in the control (P=0.0470) (Fig. 4, right). Although the COX activity after recovery (ST2) was also higher, up by 25.91% compared with the control, this difference was not significant.

The effect of cold on ATP levels

In muscles, a significant increase was observed immediately after cold treatment. The ATP level increased by approximately 53.25%, from 311.14 pmol mg^{-1} to 476.83 pmol mg^{-1} (Tukey's test, P=0.0056), compared with the control. However, after the recovery period, the ATP level substantially decreased by 57.78% to 131.37 pmol mg^{-1} (Tukey's test, P=0.0028) (Fig. 5, left). In the fat body, the ATP level significantly differed (i.e. increased) among treatments (ANOVA, P=0.0002; d.f.=2). Immediately after cold stress (ST1), the level of ATP was 25.17 pmol mg^{-1}, and after recovery, it was 42.25 pmol mg^{-1}, and the ATP level in the control sample was 12.05 pmol mg^{-1}. Thus, the level of ATP in this tissue was over 2-fold (Tukey's test, P=0.0016) and 3.5-fold (Tukey's test, P=0.0004) higher in the treated groups than in the control group, respectively (Fig. 5, right).

The effect of cold on the level of oxidative stress

Superoxide anion production

The overall generation of superoxide anion was twice as high in fat body mitochondria than in muscle mitochondria. In muscle mitochondria, a trend of a slight decrease in O_2^- generation was observed, although this change was not statistically significant (P=0.6871, d.f.=2) (Fig. 6). On the other hand, the generation of O_2^- in fat body mitochondria was strongly affected by the treatments (P=0.0321, d.f.=2). After 3×3 h of cold stress (ST1),

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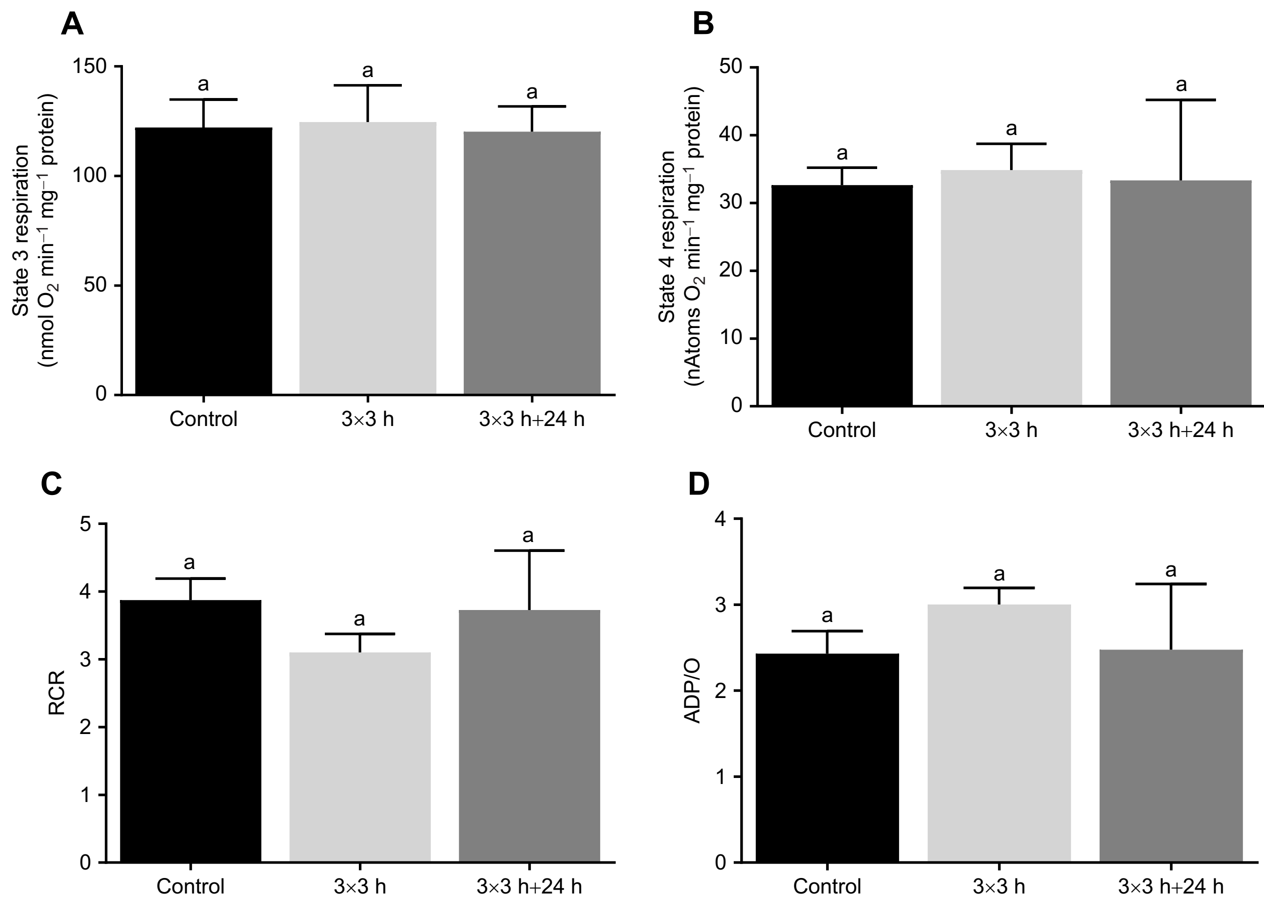

Fig. 2. Bioenergetic parameters of mitochondria isolated from the muscles of *G. coquereliana* after repeated cold stress (3×3 h) and recovery (3×3 h+24 h). (A,B) Respiration was measured in the presence of 10 mmol l^{-1} pyruvate plus 10 mmol l^{-1} malate and in the absence (state 4 respiration) or presence (state 3 respiration) of 400 µmol l^{-1} ADP. Changes in (C) the respiratory control ratio (RCR) and (D) ADP/O ratio of the muscles of *G. coquereliana* cockroaches following repeated exposure to cold and recovery. Data are means±s.e.m. (*n*=7). The same letters represent no statistically significant difference between groups. Statistical significance was determined using one-way ANOVA with Tukey's multiple comparisons test.

the generation of O_2^- was significantly reduced by 50.37% (Tukey's test, *P*=0.0338) and after the recovery phase (ST2), it returned to the control level (Tukey's test, *P*=0.6933) (Fig. 6).

Lipid peroxidation

The level of lipid peroxidation followed a decreasing trend after exposure to cold stress (3×3 h of 4°C) and the value in both tested tissues changed to similar degrees, that is, by 41.57%, from 0.558 µmol l^{-1} µg^{-1} to 0.326 µmol l^{-1} µg^{-1}, in muscle and by 42.69%, from 1.136 µmol l^{-1} µg^{-1} to 0.651 µmol l^{-1} µg^{-1}, in the fat body compared with control samples; however, these changes were not statistically significant (Tukey's test, *P*=0.2604 and *P*=0.3136, respectively). After the recovery period (3×3 h of 4°C+24 h), the lipid peroxidation level returned to the control levels in muscle tissue (0.653 µmol l^{-1} µg^{-1}); however, in the fat body, it remained low (0.751 µmol l^{-1} µg^{-1}) (Fig. 7). The overall lipid peroxidation level was 2-fold higher in fat body tissue than in muscles under control conditions (Fig. 7).

Activity of antioxidant enzymes

In muscles, the expression of the gene that encodes SOD was significantly different among the treatment groups (ANOVA, *P*=0.0384, d.f.=2); compared with the control treatment, *SOD* gene expression decreased over four times after repeated exposure to cold (Tukey's test, *P*=0.0451) and returned to baseline levels after the recovery period (Tukey's test, *P*=0.9147) (Fig. 8A). In the fat body tissue, we observed similar differences among the treatment groups (ANOVA, *P*=0.0180). After cold stress (ST1), the expression of *SOD* seemed to follow a decreasing trend, and decreased by over 50% (Tukey's test *P*=0.0625) and then increased 1.83-fold after the recovery period (Tukey's test, *P*=0.1639). Although these results were not statistically significant, *SOD* expression tended toward upregulation after recovery (ST2), especially compared with levels immediately after cold treatment (ST1) (Tukey's test, *P*=0.0141) (Fig. 8B). Next, we measured the activity and abundance of SOD in both tissues. The changes in enzyme activity in muscle tissue followed the same pattern as the changes in the protein level. After cold treatment (ST1), the SOD activity decreased by 31.98% (*P*=0.0367) compared with the control; after recovery, there was a slight increase compared with that in insects experiencing cold stress but SOD activity was still 22.20% lower than in control individuals (Fig. 8C). In the fat body, the activity of SOD was similar in both treatment groups as well as in the control group. It decreased by 18.87% in the 3×3 h group and increased by only 2.03% after recovery (ST2) (Fig. 8D). The protein level showed the opposite pattern of accumulation depending on the tissue tested. In the muscle, a slight, not statistically significant, decrease in SOD level was observed of 38.88% and 24.27% in the 3×3 h and 3×3 h+24 h treatment groups, respectively (Tukey's test, *P*=0.7506 and *P*=0.8930, respectively) (Fig. 8E and Fig. S2A),

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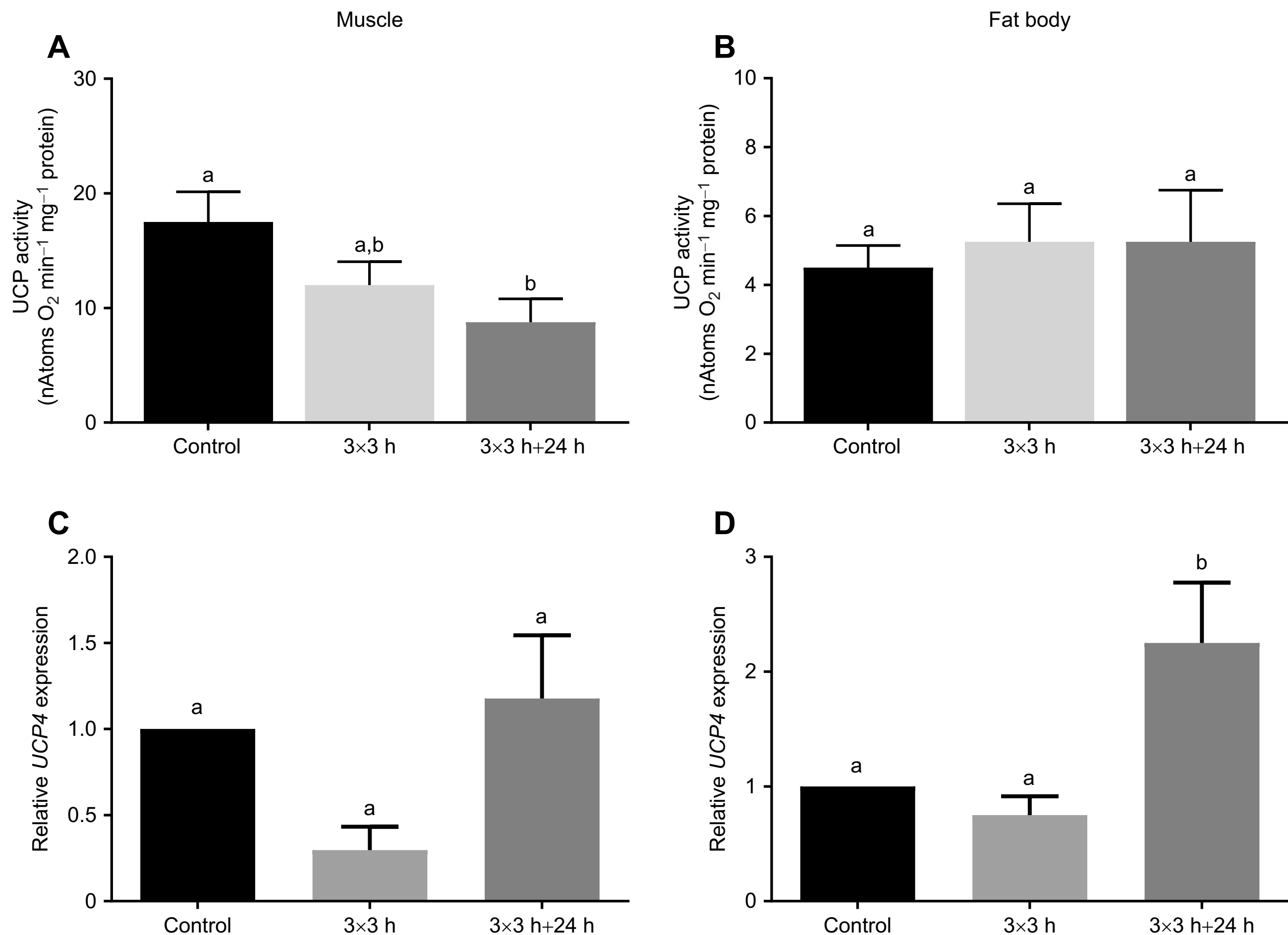

Fig. 3. Changes in the UCP activity of the muscle and the fat body of *G. coquereliana* cockroaches following repeated cold exposure (3×3 h) and recovery (3×3 h+24 h). (A,B) UCP activity in the muscles and the fat body of cockroaches, respectively. UCP activity was measured in isolated mitochondria in the presence of substrate, palmitic acid (PA, an activator of UCP) and GTP (an inhibitor of UCP). To exclude the activities of the ATP/ADP antiporter and ATP synthase, carboxyatractyloside (1.5 μmol l^{-1}) and oligomycin (1 mg mg^{-1} mitochondrial protein) were added to the incubation medium. (C,D) Changes in *UCP* expression levels in the muscles and the fat body of cockroaches, respectively. Data are means±s.e.m. (n=4 for A,B; n=6 for C,D). Different letters represent statistically significant differences between groups ($P \leq 0.05$). Statistical significance was determined using one-way ANOVA with Tukey's multiple comparisons test.

whereas the SOD level in the fat body increased 83.98% and 128.41% in the 3×3 h and the 3×3 h+24 h treatment groups, respectively (Tukey's test, P=0.6730 and P=0.4092) (Fig. 8F and Fig. S2B).

A substantial decrease in *CAT* expression was observed in muscle tissues from both experimental groups (Fig. 9A). *CAT* expression was downregulated by 6.8-fold in the 3×3 h cold group (P=0.0313) and 16.26-fold after recovery (P=0.0313). On the other hand, in the fat body, such downregulation was observed only at the first timepoint (ST1) (decrease by 2.15-fold, P=0.0313). In the 3×3 h+24 h treatment group, a significant, 2.95-fold upregulation of *CAT* was observed (Fig. 9B) (P=0.0313). Although in most cases these changes were not statistically significant, a similar trend of decreasing CAT activity was observed in both tissues. A significant decrease was observed only in muscle tissue after 24 h of recovery (P=0.0194) (Fig. 9C,D). After cold stress (3×3 h) and recovery (3×3 h+24 h), the activity of the enzyme decreased by 34.90% and 51.41%, respectively (Fig. 9C), in the muscles. Similarly, in the fat body tissue, these values were 12.38% and 22.59% lower than those in the control group, respectively (Fig. 9D). The changes in the protein level in muscles followed the same pattern as that observed for the activity, showing a decrease over experimental procedures (Fig. 9E and Fig. S3A). In the fat body, a substantial decrease in the CAT protein level was also observed, that is, CAT level was decreased by 44.78% and 47.32% in the 3×3 h and 3×3 h+24 h groups, respectively (P=0.0208 and P=0.0386, respectively) (Fig. 9F and Fig. S3B).

The effect of cold stress on the expression of HSP70

In muscle tissue, the expression of *HSP70* was affected by the cold treatments (ANOVA, P=0.0142): it slightly, but not significantly, increased 0.33-fold in the 3×3 h group (ST1) (Tukey's test, P=0.9704) and it significantly increased by 4.33-fold after recovery (ST2) (Tukey's test, P=0.0213) (Fig. 10A). At the protein level, after repeated stress, HSP70 abundance was significantly decreased by 34.54% (P=0.0465). After 24 h of recovery, the level of this protein increased by 7.05% compared with that in the other treatment groups but was still approximately 29.93% lower than that in the control insects (Fig. 10C and Fig. S4A).

In the fat body, the expression of *HSP70* after 3×3 h cold stress treatment remained at the same level as in the control; however, after recovery, it was significantly increased by 2.47-fold and 3.2-fold

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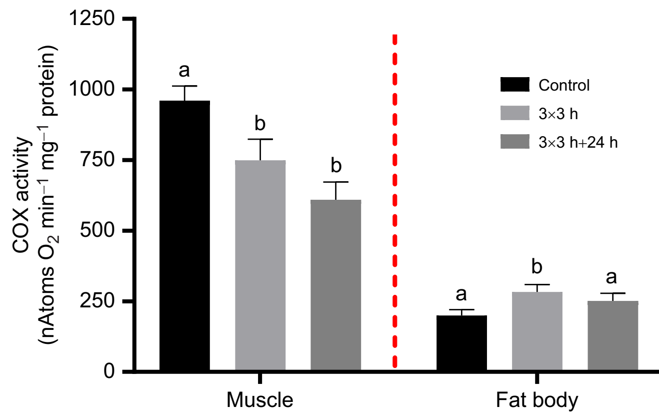

Fig. 4. Activity of cytochrome *c* oxidase in the mitochondria of the muscles and the fat body isolated from *G. coquereliana*. Data are means±s.e.m. (n=4). Different letters represent statistically significant differences in a given tissue between groups (P≤0.05). Statistical significance was determined using one-way ANOVA with Tukey's multiple comparisons test.

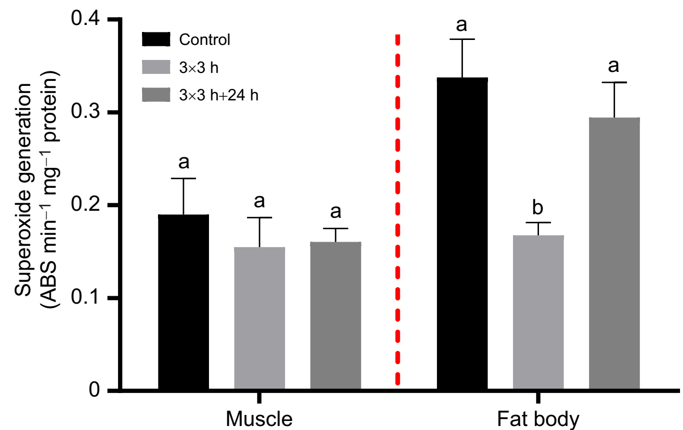

Fig. 6. Superoxide generation in mitochondria isolated from muscle and fat body tissues of *G. coquereliana* after repeated cold stress (3×3 h) and recovery (3×3 h+24 h). Data are means±s.e.m. (n=5–7). Different letters represent statistically significant differences in a given tissue between groups (P≤0.05). Statistical significance was determined using one-way ANOVA with Tukey's multiple comparisons test.

compared with the control and 3×3 h treatment groups, respectively (Tukey's test, P=0.0339 and P=0.0141, respectively) (Fig. 10B). At the protein level, the abundance of HSP70 exhibited a gradually decreasing trend, with 22.94% and 32.13% decreases in the 3×3 h and 3×3 h+24 h groups, respectively, compared with the control group; however, these decreases were not statistically significant (Fig. 10D and Fig. S4B).

DISCUSSION

The response to cold stress in many insect species from temperate or subpolar regions has been well documented. However, little is known about the cold stress response and related mechanisms in tropical species, such as cockroaches. The tropical cockroach *G. coquereliana* can withstand repeated exposure to cold (e.g. 4°C) (Chowański et al., 2017, 2015) or even partial freezing (Lubawy et al., 2019) quite well, indicating that this insect is quite well adapted to survive low temperatures. Freeze tolerance has evolved convergently in some insect groups, including Blattaria (Sinclair et al., 2003), via the development of partial freeze tolerance in species that are exposed to brief or repeated periods of cold (Sinclair et al., 2003). In the present study, we characterize the bioenergetic parameters of mitochondria that were isolated from fat body and muscle tissues as well as antioxidant capabilities in cockroaches subjected to a fluctuating thermal regime. To date, only a few studies have been conducted on mitochondria isolated from cold-stressed and/or acclimated insects (Chowański et al., 2017; Colinet et al., 2017; Da-Ré et al., 2014; Havird et al., 2020). Here, we analysed the RCR, as it is one of the most useful measures of isolated mitochondria; the RCR reflects the adjustment of the respiration rate (electron transport) to the actual demand for ATP and it indicates the degree of mitochondrial uncoupling (Brand and Nicholls, 2011). In *Drosophila melanogaster*, Colinet et al. (2017) showed that cold stress and acclimation affect these parameters. Additionally, the efficiency of oxidative phosphorylation was

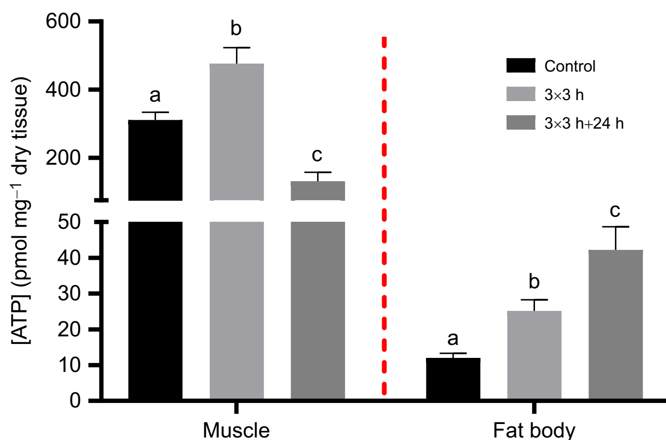

Fig. 5. ATP levels in muscles and fat body tissues of *G. coquereliana* subjected to repeated cold stress (3×3 h) and recovery (3×3 h+24 h). The data are presented as the mean value±s.e.m. (n=8). Different letters represent statistically significant differences in a given tissue between groups (P≤0.05). Statistical significance was determined using one-way ANOVA with Tukey's multiple comparisons test.

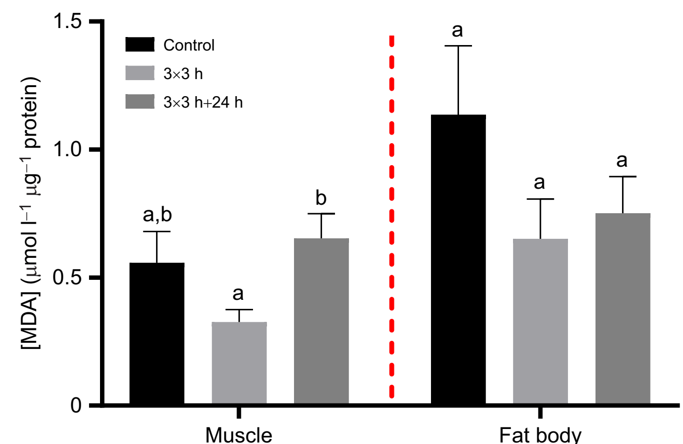

Fig. 7. Lipid peroxidation level in muscle and fat body tissues of *G. coquereliana* after being subjected to repeated cold stress (3×3 h) and recovery (3×3 h+24 h). Data are mean±s.e.m. malonaldehyde (MDA) concentrations (n=12). Different letters represent statistically significant differences in a given tissue between groups (P≤0.05). Statistical significance was determined using the Kruskal–Wallis test with Dunn's multiple comparisons test.

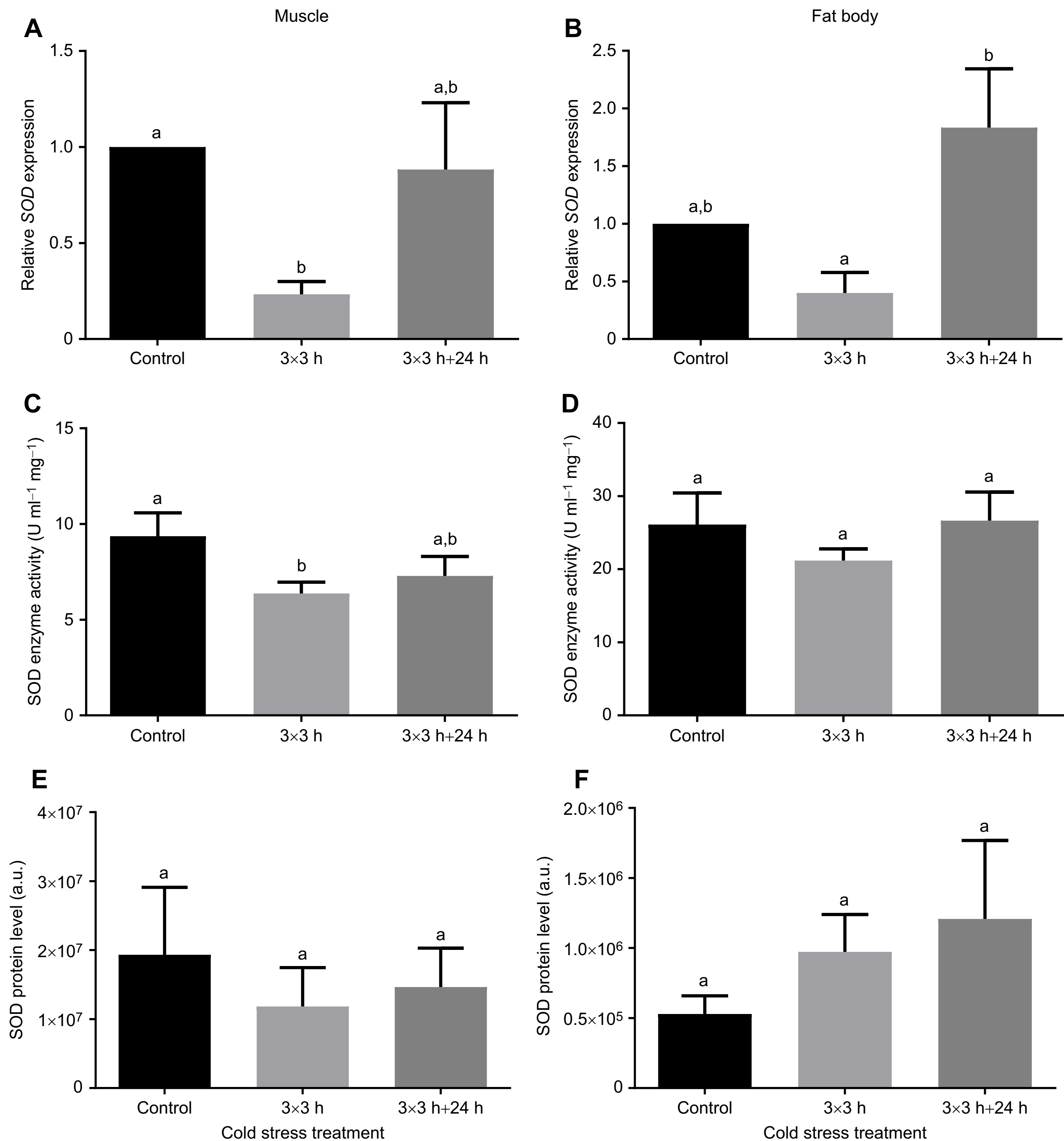

Fig. 8. The expression, activity and protein abundance of SOD in the muscle and fat body tissues of *G. coquereliana* repeatedly exposed to cold stress (3×3 h) and recovery (3×3 h+24 h). (A,B) *SOD* gene expression, (C,D) SOD enzyme activity and (E,F) SOD protein abundance in muscle (A,C,E) and fat body (B,D,F). a.u., arbitrary units. Data are means±s.e.m. (A,B,E,F: *n*=6; C,D: *n*=12). Different letters represent statistically significant differences between groups ($P \leq 0.05$). Statistical significance was determined using one-way ANOVA with Tukey's multiple comparisons test.

higher in cold-acclimated flies than that in their counterparts. Additionally, the RCR was elevated in cold-acclimated flies, which reflects a higher mitochondrial oxidative phosphorylation activity (state 3). Similarly, Havird et al. (2020) showed that in two montane mayflies, namely, *Drunella coloradensis* and *Baetis* sp., state 3 (termed OXPHOS in their study), coupling efficiency and maximal mitochondrial respiration were higher in insects that had acclimated to low temperatures. On the other hand, measuring the respiration of fly *C. costata* larvae showed that flies with freeze-sensitive (nondiapausing) phenotypes exhibited decreased oxygen consumption rates, whereas the mitochondria of freeze-tolerant insects (diapausing, cold acclimated) maintained respiratory capacity during cold stress (Štětina et al., 2020). Although these experiments were conducted at higher temperatures, which showed that the cold per se causes a decrease in oxygen consumption, the capacity to respire upon return to warm temperature is increased in

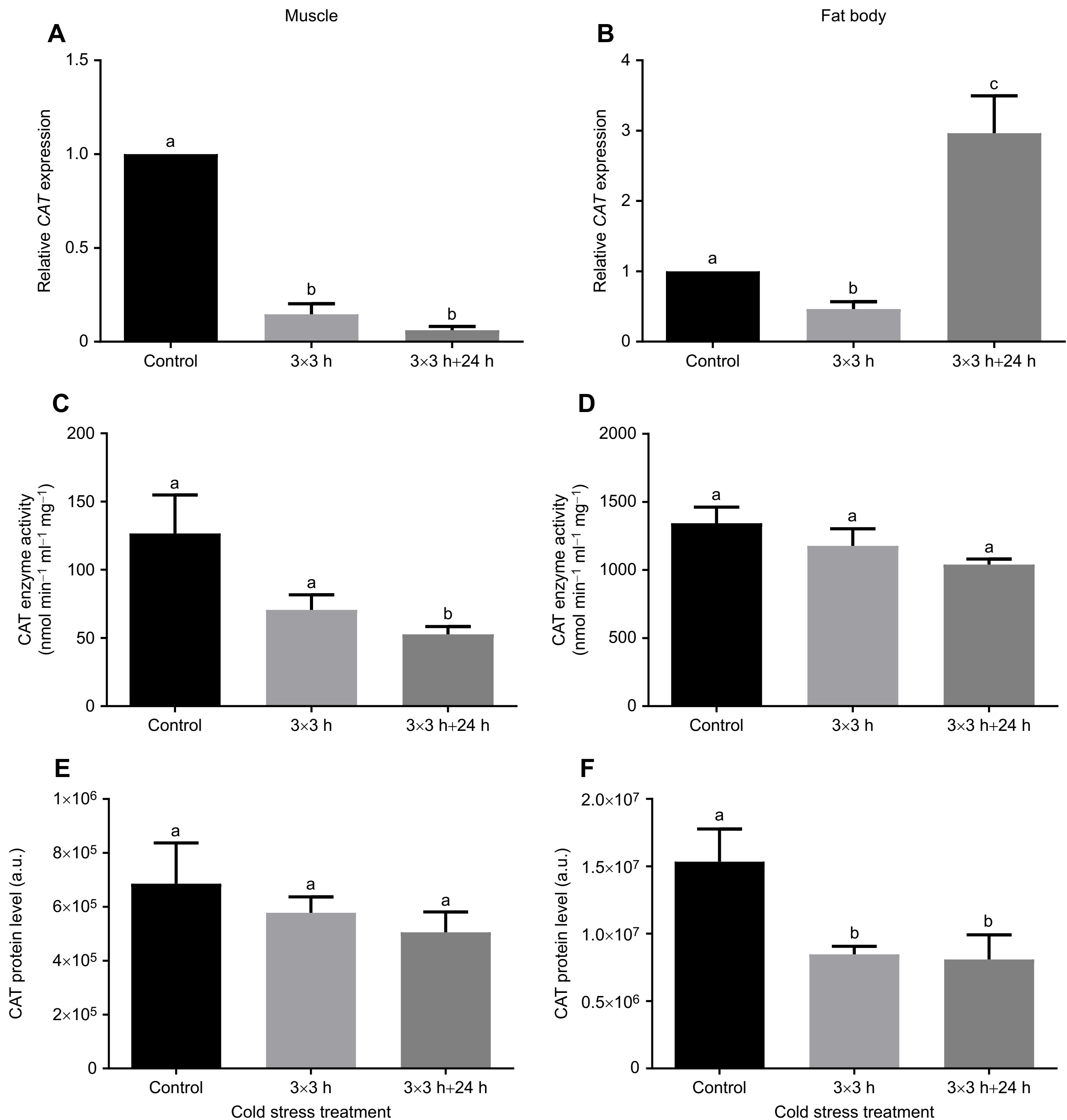

Fig. 9. The expression, activity and protein abundance of CAT in the muscle and fat body tissues of *G. coquereliana* subjected to repeated cold stress (3×3 h) and recovery (3×3 h+24 h). (A,B) *CAT* gene expression, (C,D) CAT enzyme activity and (E,F) CAT protein abundance in muscle (A,C,E) and fat body (B,D,F). Data are means±s.e.m. Different letters represent statistically significant differences between groups ($P\leq0.05$). Statistical significance was determined using one-way ANOVA with Tukey's multiple comparisons test.

cold-acclimated insects. We observed that the RCR marginally decreased in both tested tissues. This indicates that although a single exposure to cold may uncouple respiration, as we previously showed in *G. coquereliana* fat bodies (Chowański et al., 2017), the fluctuating temperatures did not induce obvious mitochondrial dysfunction, although there is no absolute RCR value that is diagnostic of dysfunctional mitochondria because RCR values are substrate and tissue dependent. Mitochondrial respiratory control is a complex function whose value depends on numerous factors, and this complexity is its main strength: a change in almost any aspect of oxidative phosphorylation changes the RCR (Brand and Nicholls, 2011). This was also observed in the trend towards an elevated ADP/O ratio in both tissues. In addition, we observed decreased UCP4 activity in muscle mitochondria (especially after recovery), which supported the value of this parameter. UCPs are mitochondrial inner membrane proteins responsible for uncoupling electron

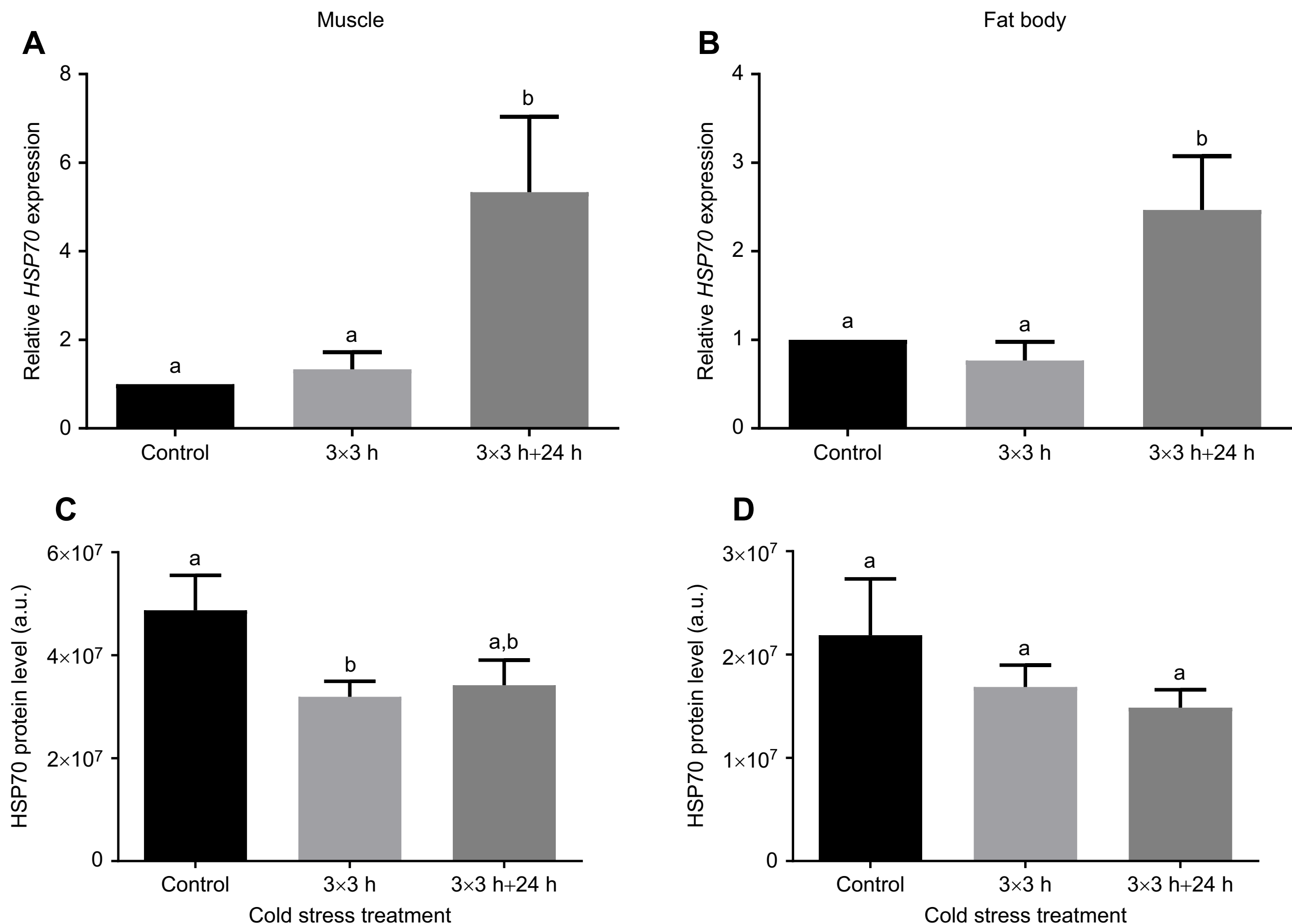

Fig. 10. Gene expression and protein level of HSP70 in the muscle and fat body tissues of *G. coquereliana* subjected to repeated cold stress (3×3 h) and recovery (3×3 h+24 h). (A,B) *HSP70* gene expression and (C,D) HSP70 protein abundance in muscle (A,C) and fat body (B,D). Data are means±s.e.m.The data are presented as the mean value±s.e.m. (n=6). Different letters represent statistically significant differences between groups (P≤0.05). Statistical significance was determined using one-way ANOVA with Tukey's multiple comparisons test.

transport through the respiratory chain from ATP synthesis, leading to a decrease in the efficiency of ATP synthesis (Sluse et al., 2006). Although we did not observe an increase in UCP activity in the fat body as previously observed during a single exposure (Chowański et al., 2017), which indicated thermogenic activity, *UCP4* was upregulated after 24 h of recovery. This may be related to the increased level of superoxide anion production that was observed in this tissue after recovery. Since UCP activation decreases ROS production, UCPs are considered an antioxidant system that counteracts oxidative damage in cells at the expense of the yield of oxidative phosphorylation (Slocinska et al., 2011). The increase in O_2^- production after recovery may be a signal that results in the higher expression of *UCP4* to produce the protein that is needed further to decrease the potentially harmful levels of ROS, whose production is elevated after cold stress (Lalouette et al., 2011). We have previously shown that in both tested tissues of *G. coquereliana*, UCPs decrease the levels of superoxide anion (Slocinska et al., 2011, 2016). Additionally, it was suggested by Alves-Bezerra et al. (2014) that in the kissing bug (*Rhodnius prolixus*) UCP4 may participate in antioxidative machinery and protect cells from the detrimental effects of ROS after cold stress. Ulgherait et al. (2020) showed that *D. melanogaster* per^{01} mutants, which exhibit constitutively high expression of *Ucp4B* and *Ucp4C*, recover after cold shock much faster than control specimens. This suggests that per^{01} mutants generate more heat than control flies do.

COX is an enzyme that is localized to the inner mitochondrial membrane (IMM) and is the final electron acceptor in the respiratory chain (Kadenbach and Hüttemann, 2015; McMullen and Storey, 2008b). Enzymes of the IMM have been shown to be repressed in insects during winter months. In both freeze-avoiding and freeze-tolerant insects, a decrease in COX activity can be observed during winter (Joanisse and Storey, 1994). In the goldenrod gall moth *Epiblema scudderiana*, COX activity decreases by approximately 30%; however, *COX1* mRNA transcript levels remain unchanged (McMullen and Storey, 2008a). Similarly, Camus et al. (2017) did not observe any changes in *COX* in *Drosophila simulans* between the test populations, even though these populations differed in cold tolerance. We found that in *G. coquereliana*, COX activity differed in the different tissues. The decrease in COX activity in muscles after cold stress was probably a consequence of the suppression of protein synthesis to reduce the amounts of selected enzymes in mitochondria during cold exposure (suppression of metabolic activity) or a consequence of the inhibition of enzyme activities, perhaps by reversible controls (McMullen and Storey, 2008b). Interestingly, in our study, COX activity was increased in the fat body after repeated cold stress. The fat body is a partial homologue of liver and adipose tissue in vertebrates. In mammals, it is well established that cold acclimation and/or chronic cold exposure cause an increase in COX activity in liver and adipose tissues (Terblanche et al., 2000). In mammalian organs, COX also acts as an antioxidant

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enzyme, and it has a 'pocket' or 'crypt' in its active site in which free radical intermediates are entrapped and retained until water is formed (Chance et al., 1979). In our study, we did not observe an increase in levels of reactive oxygen species but rather a decrease in all measured parameters. Thus, COX may participate in antioxidant defence in *G. coquereliana*. To our knowledge, this is one of the first reports to measure tissue-specific COX activity in insects after cold stress, and this topic needs further investigation.

Typically, when insects are exposed to cold stress, an increase in antioxidant activity (SOD and CAT) can be observed, which is the result of increased ROS production (Farahani et al., 2020; Gao et al., 2013; Lalouette et al., 2011). However, this was not the case in this study, as neither treatment induced oxidative stress (measured by superoxide production and lipid peroxidation), but the levels of the measured parameters decreased immediately after cold stress. We also did not observe an increase in the expression, abundance and activity of SOD and CAT, but rather we observed stable or decreasing levels depending on the treatment and/or enzyme (the only exception being *CAT* expression in the fat body after recovery). In the response of the citrus red mite *Panonychus citri* to low-temperature stress (5 and 10°C), SOD activity increased at the beginning of the stress response and then decreased (Yang et al., 2010). This is consistent with the findings of An and Choi (2010), where the early stage of exposure to acute changes in temperature resulted in oxidative stress that was regulated by antioxidant enzymes but continued stress caused by acute temperature exposure resulted in decreased SOD activity. Our results could be explained by the fact that those two enzymes are not the only enzymes in the whole antioxidant machinery. Mucci et al. (2021) showed that in cold-stressed honeybees, H_2O_2 accumulation did not increase and there was no increase in catalase activity. However, the total antioxidant activity of bees increased after cold stress, suggesting a role for nonenzymatic antioxidant compounds in controlling ROS levels (Mucci et al., 2021). The authors also determined that high H_2O_2 levels can be neutralized by peroxidases, as their level significantly increases after cold stress (Mucci et al., 2021). As we did not determine the activity of other antioxidant enzymes, it is possible that different mechanisms are responsible for the decreased superoxide and lipid peroxidation levels. Additionally, other proteins (e.g. HSP90 and vitellogenin) have been associated with tolerance to cold stress (Ramirez et al., 2017). This indicates that other antioxidant mechanisms that are induced by cold stress in cockroaches are sufficient to maintain ROS at lower levels, allowing for the survival of insects. Insects subjected to cold stress rely on two strategies to avoid oxidative stress: (1) an increase in the activity of antioxidant enzymes, as is the case with chill-susceptible (in the case of these insects, also mild uncoupling by UCP) and freeze-avoiding insects, or (2) a suppression of metabolism to reduce the production of ROS and protect cells, as observed in freeze-tolerant species (Lubawy et al., 2022). *G. coquereliana* seems to rely on the second mechanism, that is, lowering its metabolism. Since this insect can survive partial freezing (Lubawy et al., 2019), this might be an explanation. However, this leaves the door open for research on this topic in tropical insects.

Although bioenergetic parameters may indicate lower ATP synthesis through oxidative phosphorylation, here, the level of ATP in the tested tissues increased, which may seem counterintuitive. However, recovery from cold stress requires energy (Štětina et al., 2018) and the concentration of ATP can be buffered using phosphate transfer from the phosphagen pool, such as arginine phosphate in invertebrates (Ellington, 2001; Nation, 2008). Indeed, Williams et al. (2018) showed that in *D. melanogaster*, ATP concentrations are maintained during cooling without extensive reliance on anaerobic metabolism but rather on the phosphagen pool. Other observations corroborate previous observations that ATP levels do not decrease during sublethal cold exposure, and in some cases, ATP levels even increase (Colinet, 2011; El-Shesheny et al., 2016; Macmillan et al., 2012; Pullin and Bale, 1988; Pullin et al., 1990). Another possibility is that cold stress has less of an effect on ATP production than on ATP consumption, which results in the accumulation of this molecule, as suggested by Colinet (2011) in the *Alphitobius diaperinus* beetle. For this to be proven, rates of anabolism and catabolism should be quantified using, for example, calorespirometry or infrared gas analysis (Acar et al., 2001).

In most studied insects, HSPs are induced in response to cold stress (Jiang et al., 2012; Rinehart et al., 2007). It has been shown that changes can be observed in HSP70 at both the gene and protein levels during cold stress and recovery (Colinet et al., 2007, 2010a; Goto and Kimura, 1998; Kimura et al., 1998). Our previous studies showed that after a single exposure to cold, new isoforms of HSP70 protein are expressed (Chowański et al., 2015) and the protein abundance increases in muscle and fat body tissues (Chowański et al., 2017). However, in this study, we observed the opposite effect. The *HSP70* expression levels in both tissues increased, especially after 24 h recovery, while at the protein level, there was a decrease in HSP70 abundance (especially in muscle immediately after cold treatment). This might be explained by the time of observation. HSP genes are known to be highly upregulated shortly after stress and to be downregulated with time (Rinehart et al., 2007, 2000). Even though it was statistically significant, the upregulation was rather low (3- to 6-fold depending on tissue); hence, our observations are most likely reminiscent of higher levels during cold stress that do not translate into higher protein levels.

In conclusion, this work shows that the bioenergetic parameters of mitochondria that were isolated from the cockroach *G. coquereliana* do not change significantly after exposure to a FTR (Fig. S6) compared with a single exposure to stress, which we previously studied (Chowański et al., 2017); these results indicate the presence of adaptive mechanisms in this tropical insect. Additionally, during the FTR, the abundance of HSP70, together with the activity of antioxidant enzymes and the levels of ROS, decrease. Interestingly, after 24 h of recovery, redox and energy balances, especially in the fat body, are restored, which shows a substantial role of this tissue in intermediary metabolism and energy management in insects as well as in response to repeated thermal stress. Further research into the activity of enzymes related to major metabolic pathways and metabolic changes in muscle and fat body tissues may allow us to elucidate and better understand the mechanisms responsible for the cold stress response of *G. coquereliana*. Additionally, understanding the physiological processes that occur under fluctuating temperatures is becoming more desirable because, as noted by Marshall and Sinclair (2010), multiple exposures to cold induce a trade-off between survival and future reproduction.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: J.L., S.P.C., M.S.; Methodology: J.L., S.P.C., M.S.; Validation: J.L., S.C.P., M.S.; Formal analysis: J.L., S.P.C.; Investigation: J.L., S.P.C., M.S.; Resources: J.L.; Data curation: J.L., S.P.C., M.S.; Writing - original draft: J.L., S.P.C., M.S.; Writing - review & editing: J.L., S.P.C., H.C., M.S.; Visualization: J.L.; Supervision: J.L., H.C., M.S.; Project administration: J.L.; Funding acquisition: J.L.

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Data availability
All relevant data can be found within the article and its supplementary information.

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