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

3 Running title: FTR effect on mitochondria in tropical insects

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14 **Key words:** UCP, HSP, bioenergetics, ROS, insect, *Gromphadorhina coquereliana*

15
16 **Summary statement:**

17 The response of the tropical cockroach *G. coquereliana* to fluctuating thermal regimes was studied
18 with an emphasis on the bioenergetic parameters of mitochondria. Repeated exposure of *G.*

19 *coquerliana* to cold promotes mitochondrial metabolism, resulting in better mitochondrial
20 respiration.

21

22 **Abstract:**

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

42

43 1. Introduction

44 Because temperate and polar species exhibit obvious adaptations to cold temperatures, their
45 responses to cold stress have been studied in detail (Chen et al., 1987; Clark et al., 2009; Clark and
46 Worland, 2008; Czajka and Lee, 1990; Montiel et al., 1998; Teets et al., 2012). Nevertheless,
47 insects that originate in tropical or semitropical regions are not necessarily completely deficient in
48 adaptative mechanisms or characteristics that allow them to resist cold stress. Indeed, some tropical
49 species may periodically be exposed to low temperatures (e.g., at high altitudes) and therefore have
50 developed strategies to cope with such conditions (Gibert et al., 2001, Goto and Kimura, 1998).
51 For instance, some cockroaches, which are mostly of tropical origin, possess mechanisms that
52 allow them to survive cold stress (Chowanski et al., 2017; Chowanski et al., 2015; Lubawy et al.,
53 2019a). This is true of *Gromphadorhina coquereliana*, a cockroach that is endemic to the tropical
54 island of Madagascar and is capable of surviving during cold and freezing stress (Chowanski et al.,
55 2017; Chowanski et al., 2015; Lubawy et al., 2019a; Slocinska et al., 2017).

56 Tolerance to cold stress results from various adaptations; among these adaptations, adaptations of
57 the mitochondria are increasingly recognized as playing a key role (Chatterjee et al., 2022; Havird
58 et al., 2020; Lubawy et al., 2022; Menail et al., 2022). During cold stress, mitochondria in insects
59 function as cellular hubs that link metabolism, stress sensing, signalling, and cell survival
60 (Bohovych and Khalimonchuk, 2016; Breda et al., 2019; Lane, 2018; Monlun et al., 2017;
61 Vakifahmetoglu-Norberg et al., 2017), and are also targets of cold stress-associated damage
62 (Lubawy et al., 2022). To date, few studies have been conducted on the cellular bioenergetics of
63 insects after exposure to low temperatures, and the results often vary with experimental context
64 and species (Chowanski et al., 2017; Colinet et al., 2017; Da-Re et al., 2014). Exposure to low
65 temperature was shown to alter ATP levels and mitochondria-driven energetic homeostasis

66 (Colinet, 2011; Pullin and Bale, 1988); these changes may then, in turn, lead to oxidative stress if
67 the production of reactive oxygen species (ROS) overcomes the antioxidative response capabilities
68 of the mitochondria (Lubawy et al., 2022; Monaghan et al., 2009). Cold stress may decrease ATP
69 production during aerobic metabolism, thus limiting the active transport of ions below passive flux
70 (Dollo et al., 2010; Overgaard and MacMillan, 2017). Cold-induced promotion of ATP synthesis
71 was also previously reported in several studies (Colinet, 2011; Macmillan et al., 2012). Hence,
72 insects in which injury and death are related to chilling (chill-susceptible and chill-tolerant insects)
73 should ensure the maintenance of their mitochondrial function in order to maintain ATP
74 production. Cold stress also affects mitochondrial and nuclear gene expression patterns, including
75 the expression of genes that encode mitochondrial energetic machinery proteins or heat shock
76 proteins (HSPs), which act to protect and cope with the effects of stress (Ballard et al., 2007; Camus
77 et al., 2017; Colinet, 2011; Colinet et al., 2018; Da-Re et al., 2014; Dollo et al., 2010; El-Shesheny
78 et al., 2016; Joannis and Storey, 1994; Macmillan et al., 2012; Pullin et al., 1990; Ramirez et al.,
79 2021; Singh et al., 2013; Teets et al., 2020). It was suggested that mitochondria can also play a role
80 in heat dissipation by uncoupling respiration to protect the cell from the detrimental effects of cold
81 stress (Da-Re et al., 2014; Ulgherait et al., 2020). Furthermore, the role of mitochondria under cold
82 stress conditions may vary depending on the species, the climatic zone in which they naturally
83 occur, and whether they are acclimated to cold stress (for details, see Lubawy et al. (2022) and
84 references within). Depending on their survival strategy, insects fall into one of three categories:
85 (I) insects that are capable of maintaining the active transport of ions during cold stress, for which
86 sufficient ATP production is needed, and the ability to maintain ATP production most likely affects
87 cold tolerance; (II) diapausing insects, in which metabolic depression is observed, and (III) insects
88 capable of surviving when their internal fluids are frozen, i.e., “the extreme survivals” (for details
89 see (Lubawy et al., 2022) and reference within).

90 Understanding mitochondrial bioenergetic parameters under cold stress conditions is increasingly
91 considered to be an important issue (Chowański et al., 2017; Colinet et al., 2017; Havird et al.,
92 2020; Menail et al., 2022; Štětina et al., 2020). However, studies on the bioenergetics of insects
93 have often been limited due to technical constraints, resulting in a lack of knowledge about the
94 mitochondrial functions and adaptations in changing thermal environments (Menail et al., 2022).
95 To date, only a few studies have investigated mitochondrial activity in cold-stressed and/or
96 acclimated insects (Chowański et al., 2017; Colinet et al., 2017; Da-Re et al., 2014; Havird et al.,
97 2020). In *G. coquereliana*, a single exposure to cold causes significant changes in mitochondrial
98 respiration activity. After 3 h at 4°C, the oxygen consumption of isolated mitochondria in the
99 resting (state 4) and phosphorylating (state 3) states was altered. Both in muscle and fat body
100 tissues, an increase in state 4 was observed, while state 3 was significantly decreased in the fat
101 body. Moreover, cold stress induced changes in the activity of uncoupling protein 4 (UCP4) and
102 the level of heat shock protein 70 (HSP70), causing significant increases in the HSP70 levels in
103 both tissues (Chowanski et al., 2017).

104 Taking the abovementioned factors into consideration, in this work, we determined how a
105 fluctuating thermal regime (FTR), which is known to exert beneficial effects on insect thermal
106 tolerance (Colinet et al., 2018), affects mitochondrial bioenergetics, their capacity to respire among
107 return to warm conditions, and whether it evokes oxidative stress in this tropical insect. To
108 determine how the *G. coquereliana* cockroach manages energy sources to adapt to low
109 temperatures, we partially mimicked the conditions that occur in the natural environment. The
110 lowest recorded temperature in Antananarivo over the last 40 years was 2.2°C, and over this time,
111 the temperature dropped below 5°C at night for 3-4 h approximately 100 times ([http://meteo-](http://meteo-climat-bzh.dyndns.org/index.php?page=stati&id=769)
112 [climat-bzh.dyndns.org/index.php?page=stati&id=769](http://meteo-climat-bzh.dyndns.org/index.php?page=stati&id=769)). Hence, we designed our experiments to

113 simulate a few very cold nights during the winter. We repeatedly exposed *G. coquereliana*
114 cockroaches to cold (3 h of cold stress for three consecutive days) and collected samples of the fat
115 body, which is an insect tissue that is analogous to mammalian liver and adipose tissue, and leg
116 muscle tissue (highly metabolic and energy-dependent tissue) (Słocińska et al., 2011) immediately
117 after the last exposure and after 24 h of recovery (Fig. S1).

118 We assumed that the FTR would trigger adaptive mechanisms that result in undisrupted
119 mitochondrial activity and capacity to respire (compared to a single exposure to cold) (Chowanski
120 et al., 2017; Chowanski et al., 2015) and an increase in oxidative stress followed by the induction
121 of protective, antioxidant mechanisms. We hypothesized that during the recovery time (24 h), these
122 protective mechanisms would be activated to restore the appropriate energetic and redox
123 homeostasis in the insect. Hence, we determined the bioenergetic parameters of mitochondria that
124 were isolated from these tissues (phosphorylating state of respiration and coupling of the
125 mitochondria by determination of respiratory control ratio), as well as the level of oxidative stress.
126 Additionally, we measured the protein level of HSP70, which is considered the major inducible
127 chaperone protein that is involved in the cold stress response (Colinet et al., 2010b; Dumas et al.,
128 2019; Yi et al., 2018). Additionally, we measured the activity of UCP 4, which could be implicated
129 in the reduction of free radicals (Alves-Bezerra et al., 2014; Slocinska et al., 2016) or in the
130 generation of heat (Da-Re et al., 2014; Ulgherait et al., 2020), as well as the activities of major
131 antioxidant enzymes, including superoxide dismutase (SOD) and catalase (CAT). Finally, we
132 measured the total pool of ATP, which is the principal molecule for storing and transferring energy
133 (Colinet, 2011).

134 2. Materials and Methods

135 2.1. Insect rearing and tissue isolation

136 Cockroaches (*Gromphadorhina coquereliana*) were reared in continuous colonies under
137 laboratory conditions of 28°C, approximately 65% relative humidity, and a 12 h light/12 h dark
138 cycle in the Department of Animal Physiology and Development, AMU in Poznań, Poland. Food
139 (lettuce, carrots, and powdered milk) and water were provided *ad libitum* as previously described
140 (Lubawy et al., 2019b). Only adult males approximately 6 cm in length and 5.5 g in weight were
141 used in the experiments. For all the experiments, cockroaches were anaesthetized as previously
142 described (Chowanski et al., 2015); then, the insects were decapitated, and the legs were cut off.
143 The legs were washed with physiological saline (PS: 139 mM NaCl, 5 mM KCl, 4 mM CaCl₂) to
144 remove the rest of the haemolymph. For muscle (Mu) isolation, only the femur was used. The fat
145 body (FB) was isolated from the abdomen using microsurgical forceps and then washed with PS.
146 Malpighian tubules and tracheas were removed with microsurgical tweezers under a stereoscopic
147 microscope. Tissue isolation was performed on ice. All the basic reagents, were purchased from
148 Merck (Merck, Warsaw, Poland) unless stated otherwise.

149 2.2. Thermal treatments

150 Adults were subjected to low temperature stress by placing them (3-5 individuals together) in
151 plastic boxes (14 x 8 x 12 cm) with food, and then the boxes were placed in an incubation chamber
152 (MIR-154, PHCBI, Netherlands) with conditions of 4°C and approximately 65% humidity. In the
153 wild, this species can occasionally be exposed to temperatures of approximately 3-6°C for a few
154 hours at night and the average annual temperatures reach 28°C (Chowanski et al., 2015), we
155 devised a thermal regime that mimicked these conditions. The insects were exposed to cold at 4°C
156 for 3 hours per day for 3 consecutive days, and then the insects were sampled for experiments

157 immediately after the last cold exposure (sampling time 1, denoted ST 1) or at 24 h to allow them
158 to fully recover (denoted sampling time 2; ST 2) (Fig. S1). The control animals were maintained
159 under the same temperature and light conditions that were used in the primary culture (28°C, 65%
160 RH, 12 h light/12 h dark). The samples for all the experiments were collected immediately after
161 the end of the thermal treatments.

162 **2.3. Mitochondria isolation**

163 Muscle mitochondria-enriched fraction was obtained using previously described methods
164 (Chowański et al., 2017). Isolation was performed in medium containing 100 mM KCl, 50 mM
165 Tris-HCl (pH 7.4), 1 mM K₂HPO₄, and 0.2% bovine serum albumin (BSA). Muscle tissue was
166 chopped into small pieces within a minute and then homogenized in a glass-Teflon homogenizer.
167 Tissues from 3-5 individuals were pooled to generate a single sample. The homogenate was then
168 centrifuged for 10 min at 500 × g. The acquired supernatant was centrifuged for 10 min at 10,000
169 × g. The pellet, which contained the mitochondria, were centrifuged at 6,000 × g for 10 min. The
170 final mitochondrial pellet was resuspended in medium containing 0.2 M mannitol, 0.1 M sucrose,
171 10 mM Tris-HCl (pH 7.4), and 0.1 mM EDTA and stored on ice for subsequent assays.

172 The mitochondria were isolated from the fat body in medium containing 0.25 M sucrose, 1 mM
173 EDTA, 5 mM Tris-HCl and 1% BSA. The tissue was homogenized in a glass Teflon homogenizer.
174 The homogenate was then centrifuged for 10 min at 800 × g, and the collected supernatant was
175 centrifuged at 12,000 × g for 10 min. The acquired pellet was then washed twice in a medium
176 containing 0.25 M sucrose, 1 mM EDTA, and 5 mM Tris-HCl and was centrifuged at 8,000 × g for
177 10 min. The final pellet that contained the mitochondria was resuspended in the same medium.

178 All the aforementioned procedures were performed at 4°C. Mitochondrial protein concentrations
179 were determined using a Direct Detect[®] Infrared Spectrometer (Merck Millipore).

2.4. 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 of incubation medium containing 0.2 M mannitol, 75 mM sucrose, 10 mM KCl, 0.1 mM EDTA, 10 mM K₂HPO₄, and 10 mM Tris-HCl (pH 7.4). Succinate (10 mM) or pyruvate (10 mM) plus malate (10 mM) 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 nmoles) was used to calculate the ADP/O ratio. O₂ uptake values are presented in nmoles of O₂ × min⁻¹ × mg protein⁻¹.

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

2.5. 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 μM), 8 mM ascorbate, 0.06% cytochrome c, and up to 2 mM N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD), which were sequentially added. The maximal O₂ consumption by COX (Complex IV) was determined by measuring the rate of its

204 consumption following the addition of TMPD. The experiments were performed with four
205 biological replicates per thermal treatment and control; each replicate consisted of mitochondria
206 that were isolated from five pooled individuals.

207 **2.6. Superoxide level**

208 The level of O_2^- released from mitochondria was determined by measuring the rate of nitroblue
209 tetrazolium (NBT) reduction by isolated mitochondria. NBT was added at a final concentration of
210 $0.07 \text{ mg} \times \text{mL}^{-1}$, and its reduction rate was measured at 560 nm (UV 1602, Shimadzu).
211 Mitochondria (0.6 mg) were incubated in 1.2 mL of incubation medium in the presence of 10 mM
212 succinate (fat body) and malate and pyruvate (muscle), which were added after the addition of
213 NBT.

214 **2.7. Isolation of mRNA and Generation of cDNA**

215 Isolated tissues (fat body or muscles) were transferred to 300 μL of RNA lysis buffer (Zymo
216 Research, Irvine, CA, USA) and homogenized for 2 minutes using a pellet homogenizer (Kimble
217 Chase, USA). For each treatment and control, at least three biological replicates were used, each
218 consisting of tissues that were collected from three pooled individuals. The homogenized tissues
219 were immediately frozen in liquid nitrogen and then stored at -80°C . Total RNA was extracted
220 using the Insect RNA MicroPrep™ Kit (Zymo Research Corp., Irvine, CA, USA) according to the
221 manufacturer's protocols. The protocol included in-column DNase I treatment to remove traces of
222 gDNA and prevent contamination of the RNA samples. ReverAid™ Reverse Transcriptase
223 (Fermentas, Waltham, MA, USA) was used to generate cDNA according to the manufacturer's
224 protocols. Quantitative real-time PCR (RT-qPCR) was performed on a Corbett Research RG-6000
225 Real Time PCR Thermocycler (QIAGEN, Venlo, Netherlands) with a Sensitive RT HSPCR Mix
226 SYBR® kit (A&A Biotechnology s.c, Gdańsk, Poland). In addition to biological replicates, at least

227 two technical replicates were performed. The expression level of the gene encoding *G.*
228 *coquereliana* 18S rRNA was used as a control. To check for potential foreign contamination of
229 samples, “no template” negative controls (DNA/RNA free water) were tested. The relative
230 expression was calculated using the $2^{\Delta\Delta Ct}$ method. The primers used for specific genes were as
231 follows; *18S rRNA* forward primer: CAGGGTTCGATTCCGGAGAG, *18S rRNA* reverse primer:
232 TGCTCATTCCGATTACGGGG, *UCP4* forward primer: CCGAACATGCAAAGAGCAGC,
233 *UCP4* reverse primer: AGCCACTAGTCCAGCTACCA, *SOD* forward primer:
234 TGTCCGGTCTAGGAAAAGGC, *SOD* reverse primer: GGGTTGAAATGAGCACCAGC, *CAT*
235 forward primer: TTCAGTTACGCCGACACACA, *CAT* reverse primer:
236 CCACTTCTCCTGATGGTGGG, *HSP70* forward primer: AGACACTCATCTTGGCGGTG,
237 *HSP70* reverse primer: ACAGTGTTTCGCTTAGCCCTC.

238 **2.8. Determination of protein content in fat body and muscle tissue**

239 After tissue collection, the samples were homogenized in physiological saline and centrifuged
240 at $10\,000 \times g$ for 10 min. The supernatant was used to determine the protein content in the soluble
241 fraction. The measurement was conducted with a Direct Detect1 Infrared Spectrometer (Merck
242 Millipore, Warsaw, Poland). A total of 2 μ L of supernatant was placed on the PTFE membrane and
243 left to dry for 3 min. The protein content was then measured. Bovine serum albumin (Merck
244 Millipore, Warsaw, Poland) was used as a standard. Samples were snap frozen in liquid nitrogen
245 and stored at -80°C for further analyses.

246 **2.9. Immunodetection**

247 The soluble protein fraction was resuspended in sample buffer. SDS–PAGE (using a 14%
248 polyacrylamide resolving gel) using 40 μ g of protein per well followed by Western blotting were
249 performed as previously described (Chowanski et al., 2015). Rabbit polyclonal primary antibodies

250 against Heat Shock Protein 70 (HSP 70, AS09592 from Agrisera, Vännäs, Sweden), Catalase
251 (CAT, ab16731 from Abcam, Cambridge, UK) and Superoxide dismutase 1 (SOD, ab13498 from
252 Abcam, Cambridge, UK) were used at 1:500 dilution, 1:300 dilution and 1:250 dilution,
253 respectively. The blots that were used for the immunodetection of HSP70 and SOD were incubated
254 with primary antibodies for 90 minutes, whereas those that were used for the immunodetection of
255 CAT were incubated with antibodies overnight. Goat anti-rabbit secondary antibodies conjugated
256 with horseradish peroxidase (Agrisera, AS09602) were used at dilutions of 1:10 000, 1:25 000 and
257 1:25 000, respectively, and incubated with the membranes for 1 h at room temperature. Antibodies
258 against α -tubulin at a 1:500 dilution were used as a loading control. The protein bands were
259 visualized using an Amersham ECL system and digitally quantified using ImageLab software (Bio-
260 Rad, Warsaw, Poland).

261 **2.10. ATP level determination**

262 The total amount of ATP in analysed tissues was measured with a fluorescence assay
263 (Molecular Probes ATP kit; A22066, Thermo Fisher Scientific, Warsaw, Poland) according to the
264 method that was described previously by Tennessen et al. (2014) with some modification. The
265 analysed tissues (muscles and fat body) were isolated directly after the ST1 and ST2 treatments.
266 The isolation was performed on ice, and the samples were placed in 1.5 mL Eppendorf tubes and
267 kept on ice. Then, the samples were dried to a stable weight at 60°C under a vacuum (-0.9 atm) and
268 weighed to determine the dry weight of the sample. In the next step, the samples were homogenized
269 in 300 μ L of 2.5% TCA solution and centrifuged for 5 min (4°C, 1,400 rpm). The supernatant was
270 transferred to new tubes, boiled for 5 min at 90°C and centrifuged at 12,000 rpm at 4°C for 15 min.
271 The obtained supernatant was transferred to a new tube, frozen in liquid nitrogen and stored at –
272 80°C until analysis. Prior to ATP level measurement, the ATP reaction mix was prepared according

273 to the manufacturer's protocol. The samples were diluted with buffer (25 mM Tris, 100 μ M EDTA,
274 pH 7.8) 10- or 750-fold in the case of fat body and muscles, respectively. Next, 70 μ L of sample
275 was transferred to individual wells of a white, opaque 96-well plate and mixed with 70 μ L of ATP
276 reaction mix containing luciferase. The luminescence was measured with a Synergy H1 Hybrid
277 MultiMode microplate reader (BioTek, Winooski, Vermont, USA). The measurement was initiated
278 15 sec after the reaction mix was added. The amount of ATP is present as picomoles of ATP per
279 mg of dry tissue.

280 **2.11. Lipid peroxidation**

281 The lipid peroxidation level was determined by measuring thiobarbituric acid reactive
282 substances (TBARS) using a TBARS assay kit (700870, Cayman Chemical, Tallinn, Estonia)
283 according to the manufacturer's manual. Briefly, 100 μ L of sample that was obtained as described
284 in Section 2.8 was mixed with 100 μ L of 10% trichloroacetic acid (TCA) and 800 μ L of colour
285 reagent (thiobarbituric acid (TBA), acetic acid, sodium hydroxide) in a 2 mL vial. Next, the vials
286 were placed in vigorously boiling water. After 1 hour, the vials were placed on ice and incubated
287 for 10 min to stop the reaction. After centrifugation at $1600 \times g$ at 4°C for 10 min, the absorbance
288 of the samples was measured at 530 nm using a Synergy H1 Hybrid Multi-Mode Microplate Reader
289 (BioTek, USA). The results were calculated from the standard curve of malonaldehyde (MDA) and
290 are presented as μM of MDA per μg of protein.

291 **2.12. Determination of antioxidant enzymatic activity**

292 To determine the activity of superoxide dismutase (SOD) and catalase (CAT), we used a
293 Superoxide Dismutase Assay Kit (706002, Cayman Chemical, Tallinn, Estonia) and Catalase
294 Assay Kit (707002, Cayman Chemical, Tallinn, Estonia) according to the manufacturer's
295 protocols, respectively. Both enzymes form a crucial part of the cellular antioxidant mechanism.

296 Briefly, to measure SOD activity, 10 μ L of sample was combined with 200 μ L of radical detector
297 (tetrazolium salt diluted in 50 mM Tris-HCl, pH=8.0 containing 0.1 mM
298 diethylenetriaminepentaaceticacid (DPTA) and 0.1 mM hypoxanthine) in a 96-well microplate. To
299 initiate the reaction, 20 μ L of xanthine oxidase was added to each well and incubated for 30 min
300 at room temperature on a shaker. After incubation, the absorbance of the samples was read at 540
301 nm using a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek, USA). The results were
302 calculated from the standard curve of bovine erythrocyte SOD and are presented as the unit of SOD
303 activity per millilitre per μ g of protein (U/mL/ μ g). For the CAT activity, 20 μ L of the samples
304 were mixed with 100 μ L of assay buffer (100 mM potassium phosphate, pH = 7.0) and 30 μ L of
305 methanol in a 96-well plate. To initiate the reaction, 20 μ L of hydrogen peroxide was added and
306 incubated for 20 min on a shaker. Then, 30 μ L of potassium hydroxide was added to terminate the
307 reaction, and 30 μ L of catalase purpald was added. After 10 min of incubation, catalase potassium
308 periodate was added to each well and incubated for 5 min at room temperature. The absorbance of
309 the samples was measured at 540 nm using a Synergy H1 Hybrid Multi-Mode Microplate Reader
310 (BioTek, USA). The results were calculated from the standard curve of formaldehyde and are
311 presented as nmol/min/ml/ μ g of protein.

312 **2.13. Statistical analysis**

313 For statistical analysis, we used GraphPad Prism software ver. 9 (GraphPad Software, San
314 Diego, CA, USA) (Department of Animal Physiology and Development AMU licence). Before
315 statistical analysis, the normality of the distribution (the Shapiro–Wilk test) was evaluated. All the
316 data are presented as the mean values \pm s.e.m. of the indicated number of replicates (*n*). The
317 statistical significance of differences between values of control insects and values of those exposed
318 to thermal stress were determined using one-way ANOVA with Tukey’s post hoc test (for data

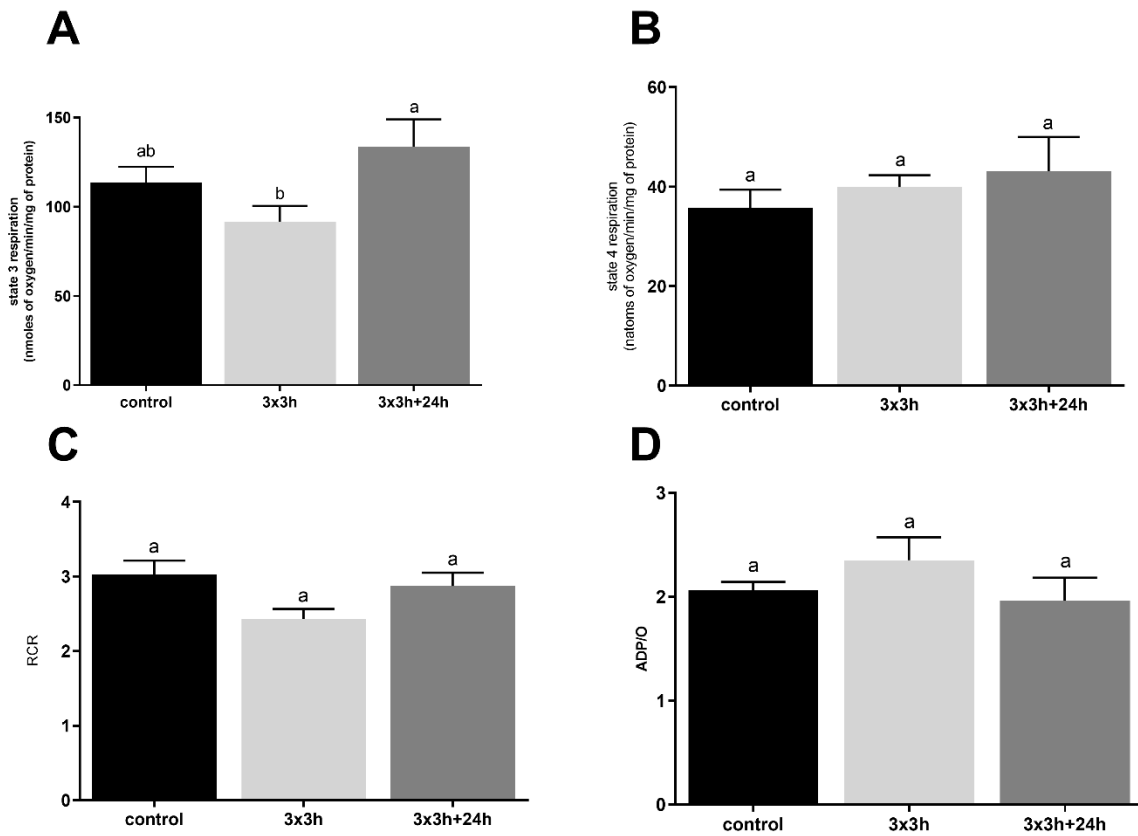
319 with normal distribution), Kruskal–Wallis test with Dunn’s post hoc test (for data without normal
320 distribution) or Student’s *t* test. Differences were considered statistically significant if $p \leq 0.05$ (*),
321 $p \leq 0.01$ (**), or $p \leq 0.001$ (***)).

322 **3. Results**

323 **3.1 The effect of cold on activity and coupling of mitochondria**

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

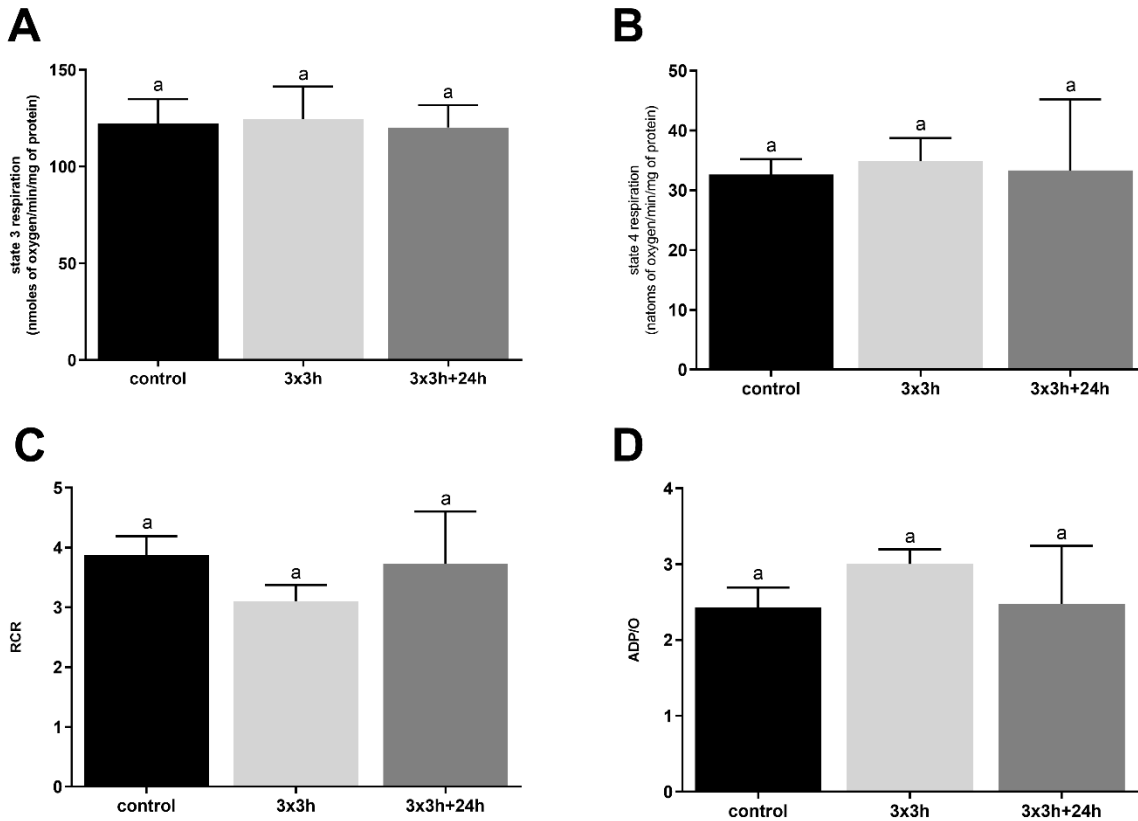
Fat Body



337

338 **Figure 1. Bioenergetic parameters of mitochondria isolated from the fat body tissue of *G.***
339 ***coquereliana*.** (A-B). Respiration was measured in the presence of 10 mM succinate and in the
340 absence (state 4 respiration) or presence (state 3 respiration) of 400 μ M ADP. C) RCR refers to
341 the respiratory control ratio. D) Changes in the ADP/O ratio of the fat body of *G. coquereliana*
342 cockroaches following repeated exposure to cold and recovery. The data are presented as the
343 mean value \pm s.e.m. ($n=7$). Different letters represent statistically significant differences between
344 groups ($p \leq 0.05$). Statistical significance was determined using one-way ANOVA with Tukey's
345 multiple comparisons test.

Muscle



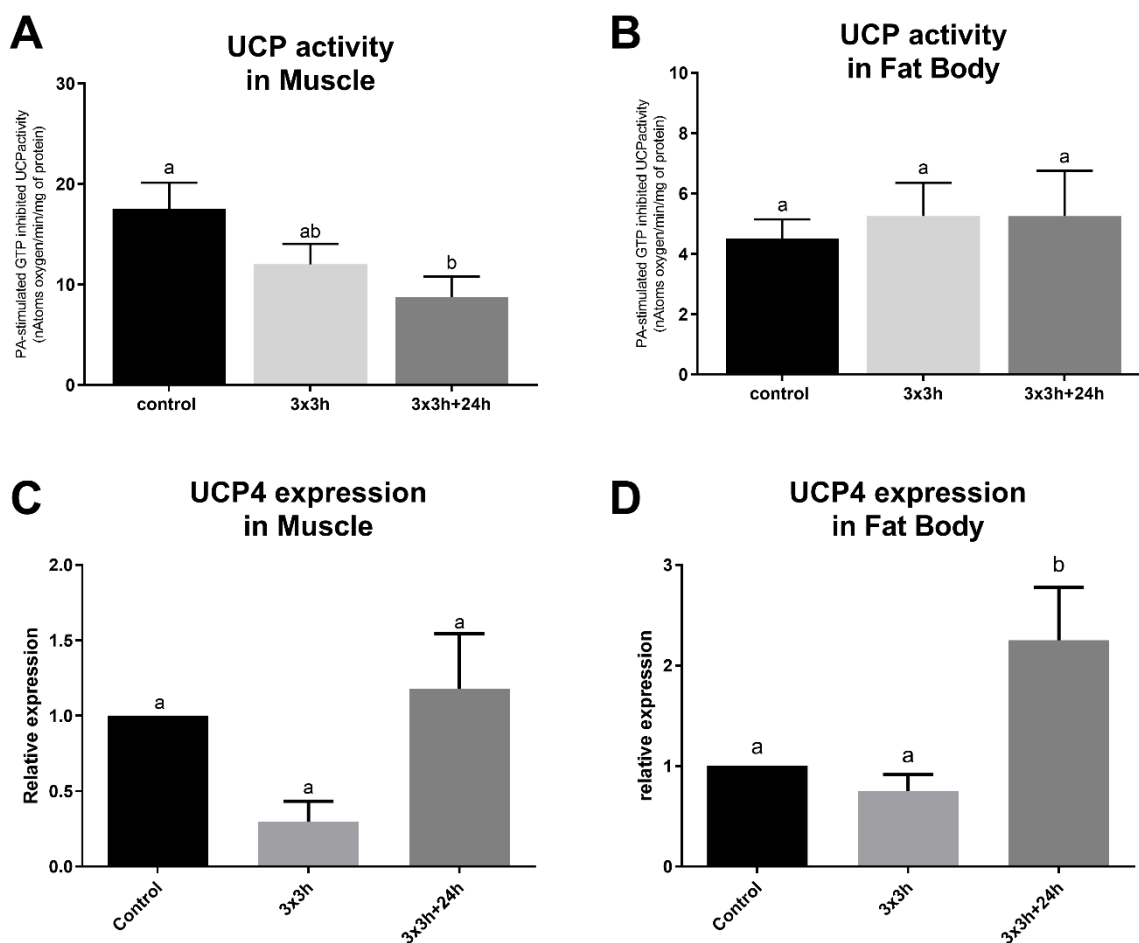
346

347 **Figure 2. Bioenergetic parameters of mitochondria isolated from the muscles of *G.***
 348 ***coquereliana*.** (A-B) Respiration was measured in the presence of 10 mM pyruvate plus 10 mM
 349 malate and in the absence (state 4 respiration) or presence (state 3 respiration) of 400 μ M ADP.
 350 C) RCR refers to the respiratory control ratio. D) Changes in the ADP/O ratio of the muscles of
 351 *G. coquereliana* cockroaches following repeated exposure to cold and recovery. The data are
 352 presented as the mean value \pm s.e.m. ($n=7$). The same letters represent no statistically significant
 353 difference between groups. Statistical significance was determined using one-way ANOVA with
 354 Tukey's multiple comparisons test.
 355

356 The activity of UCP in muscle mitochondria was three times higher than that in fat body
 357 mitochondria (Fig. 3A vs. 3B). After repeated exposure to cold (3 x 3 h) and a recovery phase (3 x
 358 3 h + 24 h), the UCP activity in muscle was lower compared to the control, especially after the ST
 359 2 treatment (Tukey's test: $p = 0.0395$) (Fig. 3A). The level of *UCP4* in muscle exhibited a

18

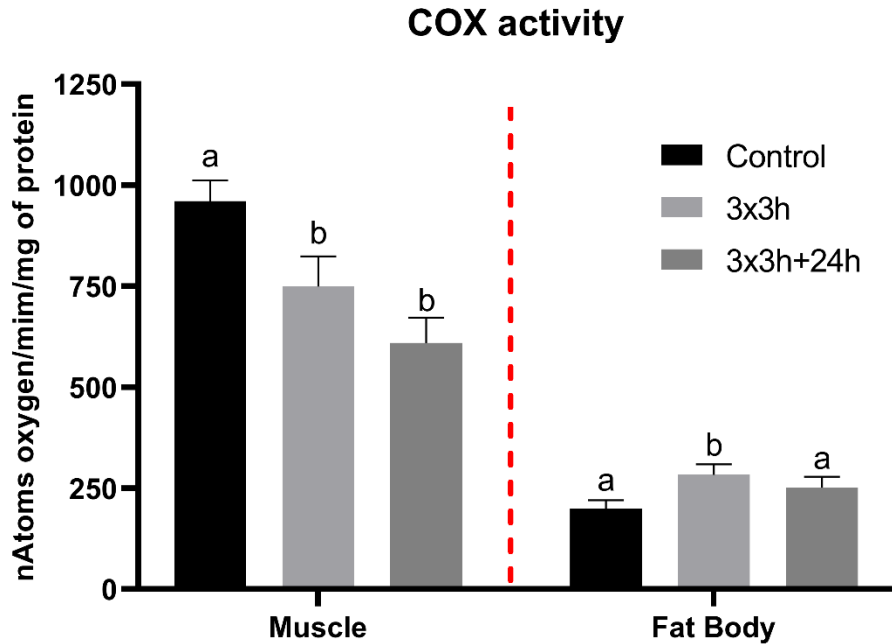
360 decreasing trend at the first timepoint (Tukey's test: $p = 0.1344$) and an increasing trend at the
 361 second timepoint compared with the control (Tukey's test: $p = 0.08515$) (Fig. 3C). However, no
 362 statistically significant changes were observed. In the fat body, UCP activity marginally increased
 363 in both variants, but the changes were not significant ($p = 0.7805$; $df = 2$) (Fig. 3B). The expression
 364 level of *UCP4* significantly varied among the 3 conditions ($p = 0.0099$; $df = 2$). Values were
 365 comparable to that of the control at the ST 1 timepoint, but it significantly increased (Tukey's test;
 366 $p = 0.0354$) by 1.83-fold after recovery (ST 2) (Fig. 3D).



367
 368 **Figure 3. Changes in the UCP activity of the muscle (A) and the fat body (B) of *G.***
 369 ***coquereliana* cockroaches following cold exposure and recovery.** UCP activity was measured
 370 in isolated mitochondria in the presence of substrate, palmitic acid (PA, an activator of UCP) and

371 GTP (an inhibitor of UCP). To exclude the activities of the ATP/ADP antiporter and ATP
372 synthase, carboxyatractyloside (1.5 μ M) and oligomycin (1 mg per 1 mg of mitochondrial
373 protein) were added to the incubation medium. Panels C and D present changes in *UCP*
374 expression levels in the muscles and the fat body of cockroaches, respectively. The data are
375 presented as the mean value \pm s.e.m. ($n=4$ for Panels A and B, $n=6$ for Panels C and D).
376 Different letters represent statistically significant differences between groups ($p \leq 0.05$).
377 Statistical significance was determined using one-way ANOVA with Tukey's multiple
378 comparisons test.

379
380 In the muscles, we observed a significant decrease in COX activity among the treatment groups
381 (ANOVA $p = 0.0116$; $df= 2$) (Fig. 4, left part). Immediately after cold stress (3 x 3 h), the COX
382 activity decreased by 22.02% (Tukey's test: $p = 0.0121$), and a further decrease of 36.52% was
383 observed after the recovery phase (Tukey's test: $p = 0.0094$) compared to the control. The
384 mitochondria that were isolated from the fat body exhibited significant changes in COX only at ST
385 1, and this value was 41.80% higher than that in the control ($p = 0.0470$) (Fig. 4, right part).
386 Although the COX activity at ST 2 was also higher, by 25.91% compared to the control, this
387 difference was insignificant.



388

389 **Figure 4. Activity of cytochrome c oxidase in the mitochondria of the muscles and the fat**
 390 **body isolated from *G. coquereliana*.** The data are presented as the mean value \pm s.e.m. ($n=4$).
 391 Different letters represent statistically significant differences in a given tissue between groups (p
 392 ≤ 0.05). Statistical significance was determined using one-way ANOVA with Tukey's multiple
 393 comparisons test.

394

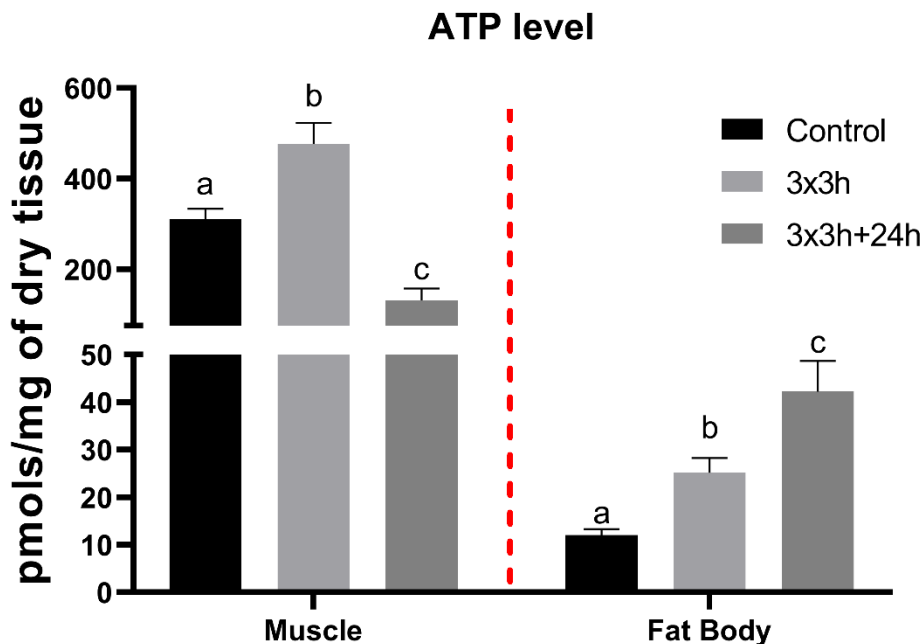
395

396 **3.2. The effect of cold on ATP levels**

397 In muscles, a significant increase was observed immediately after cold treatments. The ATP level
 398 increased by approximately 53.25%, from 311.14 pmol/mg to 476.83 pmol/mg (Tukey's test $p =$
 399 0.0056), compared to the control. However, after the recovery period, the ATP level substantially
 400 decreased by 57.78% to 131.37 pmol/mg (Tukey's test, $p = 0.0028$) (Fig. 5, left part). In the fat
 401 body, the ATP level significantly differed (i.e., increased) among treatments (ANOVA $p= 0.0002$;
 402 $df= 2$). Immediately after cold stress, the level of ATP was 25.17 pmol/mg, and after recovery, it
 403 was 42.25 pmol/mg, and the ATP level in the control sample was 12.05 pmol/mg. Thus, the level

21

404 of ATP in this tissue was over 2-fold (Tukey's test, $p = 0.0016$) and 3.5-fold (Tukey's test, $p =$
405 0.0004) higher in the treated groups than in the control group, respectively (Fig. 5 right part).



406
407 **Figure 5. ATP levels in muscles and fat body tissues of *G. coquereliana* subjected to**
408 **repeated cold stress (3 x 3 h) and after recovery (3 x 3 h + 24 h).** The data are presented as the
409 mean value \pm s.e.m. ($n = 8$). Different letters represent statistically significant differences in a
410 given tissue between groups ($p \leq 0.05$). Statistical significance was determined using one-way
411 ANOVA with Tukey's multiple comparisons test.

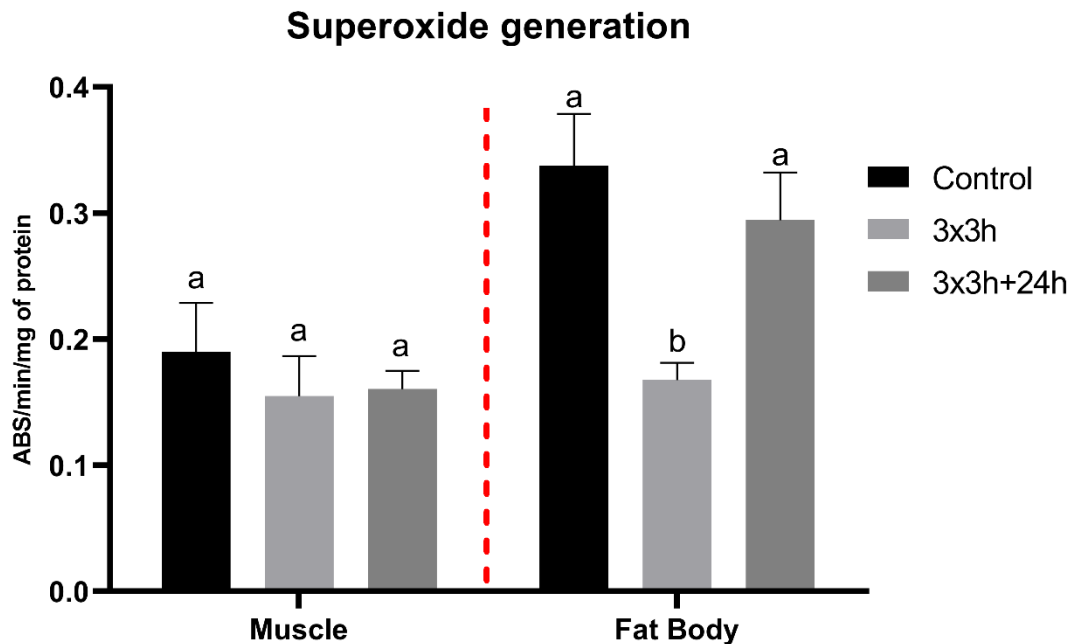
412

413 3.3. The effect of cold on the level of oxidative stress

414 3.2.1. Superoxide anion production

415 The overall generation of superoxide anion was twice as high in fat body mitochondria than in
416 muscle mitochondria. In muscle mitochondria, a trend of a slight decrease in O_2^- generation was
417 observed, although this change was not statistically significant ($p = 0.6871$, $df=2$) (Fig. 6). On the
418 other hand, the generation of O_2^- in fat body mitochondria was strongly affected by the treatments

419 ($p = 0.0321$, $df=2$). After 3 x 3 h of cold stress, the generation of O_2^- was significantly reduced by
420 50.37% (Tukey's test, $p = 0.0338$), and after the recovery phase, it returned to the control level
421 (Tukey's test, $p = 0.6933$) (Fig. 6).



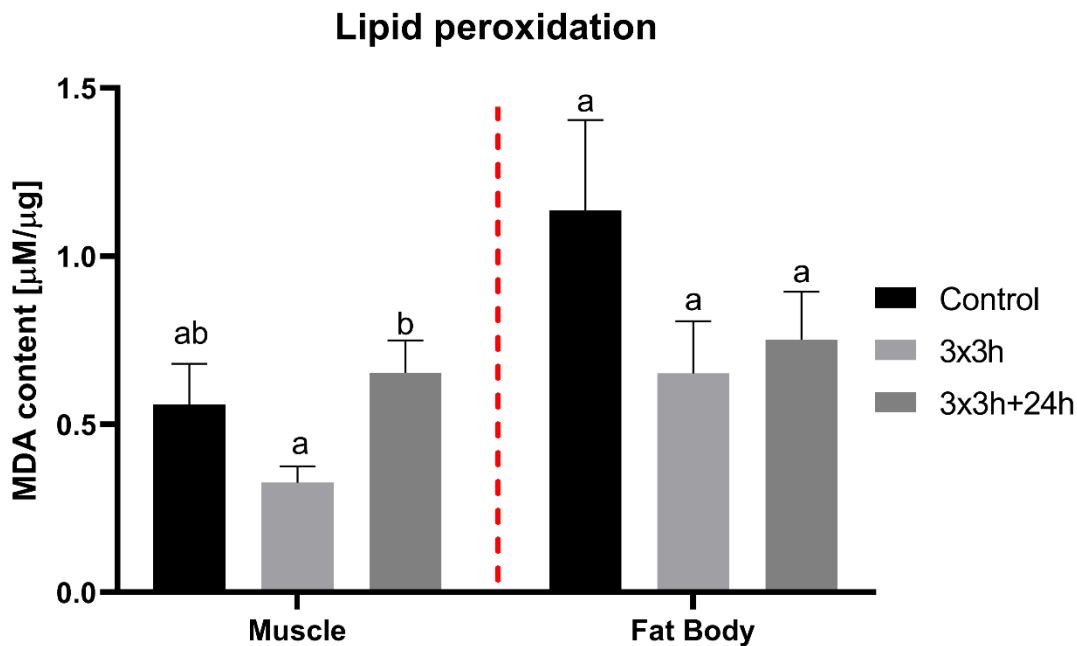
422
423 **Figure 6. Superoxide generation in mitochondria that were isolated from muscle and fat**
424 **body tissues of *G. coquereliana* after repeated cold stress (3 x 3 h) and recovery (3 x 3 h + 24**
425 **h).** The data are presented as the mean value \pm s.e.m. ($n=5-7$). Different letters represent
426 statistically significant differences in a given tissue between groups ($p \leq 0.05$). Statistical
427 significance was determined using one-way ANOVA with Tukey's multiple comparisons test.

428

429 3.2.2. Lipid peroxidation

430 The level of lipid peroxidation seemed to follow a decreasing trend after exposure cold stress
431 (3 x 3 h of 4°C), and the value in both tested tissues seemed to change to similar degrees, that is,
432 by 41.57%, from 0.558 $\mu\text{M}/\mu\text{g}$ to 0.326 $\mu\text{M}/\mu\text{g}$, in muscle and by 42.69%, from 1.136 $\mu\text{M}/\mu\text{g}$ to
433 0.651 $\mu\text{M}/\mu\text{g}$, in the fat body compared to control samples; however, these changes were not

434 statistically significant (Tukey's test, $p = 0.2604$ and $p = 0.3136$, respectively). After the recovery
435 period (3 x 3 h of 4°C + 24 h), the lipid peroxidation level returned to the control levels in muscle
436 tissue (0.653 $\mu\text{M}/\mu\text{g}$); however, in the FB, it remained low (0.751 $\mu\text{M}/\mu\text{g}$) (Fig. 7). The overall
437 lipid peroxidation level was 2-fold higher in fat body tissue than in muscles under control
438 conditions (Fig. 7).

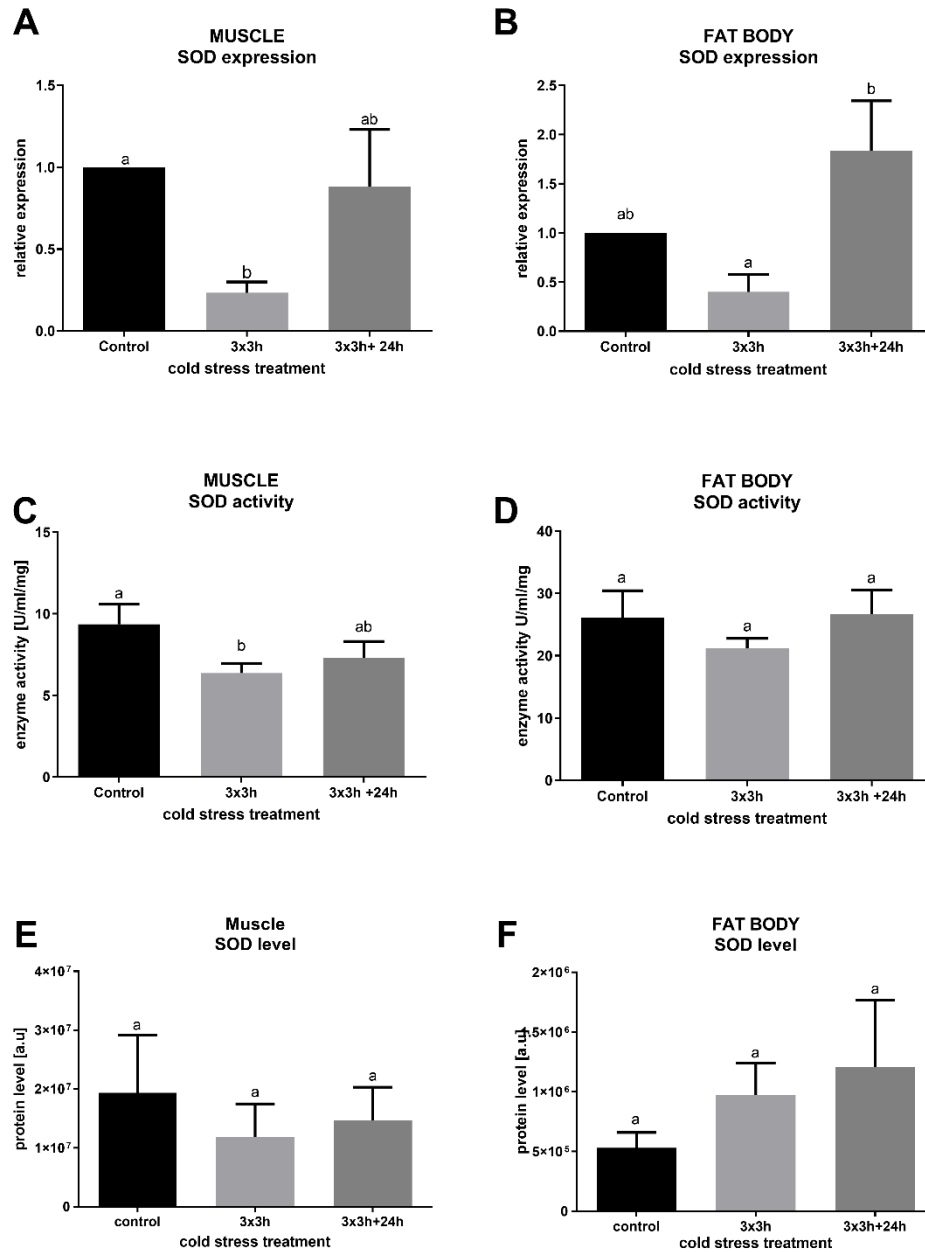


439
440 **Figure 7. Lipid peroxidation level in muscle and fat body tissues of *G. coquereliana* after**
441 **being subjected to repeated cold stress (3 x 3 h) and after recovery (3 x 3 h + 24 h).** The data
442 are presented as the mean value \pm s.e.m. (n=12). Different letters represent statistically significant
443 differences in a given tissue between groups ($p \leq 0.05$). Statistical significance was determined
444 using the Kruskal–Wallis test with Dunn's multiple comparisons test.

445 3.2.3. Activity of antioxidant enzymes

446 In muscles, the expression of the gene that encodes SOD was significantly different among the
447 treatment groups (ANOVA, $p = 0.0384$, $df=2$); compared with the control treatment, *SOD* gene
448 expression decreased by over four-fold after repeated exposure to cold (Tukey's test, $p = 0.0451$)

449 and returned to baseline levels after the recovery period (Tukey's test, $p = 0.9147$) (Fig. 8A). In
450 the fat body tissue, we observed similar differences among the treatment groups (ANOVA, $p =$
451 0.0180). After cold stress (ST 1), the expression of *SOD* seemed to follow a decreasing trend; it
452 decreased by two and a half times at ST 1 (Tukey's test $p = 0.0625$) and then increased by 1.83-
453 fold after the recovery period (Tukey's test, $p = 0.1639$). Although these results were not
454 statistically significant, *SOD* expression trended toward upregulation at ST 2, especially compared
455 with ST 1 (Tukey's test, $p = 0.0141$) (Fig. 8B). Next, we measured the activity and abundance of
456 *SOD* in both tissues. The changes in enzyme activity in muscle tissue followed the same pattern as
457 the changes in the protein level. At ST 1, the *SOD* activity decreased by 31.98% ($p = 0.0367$)
458 compared to the control, and after recovery, a slight increase was observed compared to that in
459 insects experiencing cold stress; however, *SOD* activity was 22.20% lower than in control
460 individuals (Fig. 8C). In the fat body, the activity of *SOD* was similar in both treatment groups as
461 well as in the control group. It tended to decrease by 18.87% in the 3 x 3 h group and increase by
462 only 2.03% in the 3 x 3 h + 24 h group (Fig. 8D). The protein level showed the opposite pattern of
463 accumulation depending on the tissue tested. In the muscle, a slight, not statistically significant,
464 decrease in *SOD* level was observed, by 38.88% and 24.27% in the 3 x 3 h and 3 x 3 h + 24 h
465 treatment groups, respectively (Tukey's test, $p = 0.7506$ and $p = 0.8930$, respectively) (Fig. 8E and
466 Fig. S2A), whereas *SOD* level in the FB exhibited increasing trends of 83.98% and 128.41% in the
467 3 x 3 h and the 3 x 3 h + 24 h treatment groups (Tukey's test, $p = 0.6730$ and $p = 0.4092$) (Fig. 8F
468 and Fig. S2B).



469

470 **Figure 8. The expression (A and B), activity (C and D) and protein abundance (E and F)**
 471 **of the SOD enzyme in the muscle (A, C and E) and fat body (B, D and F) tissues of *G.***

472 ***coquereliana* repeatedly exposed to cold stress (3 x 3 h) and recovery (3 x 3 h + 24 h). The**
 473 **data are presented as the mean value \pm s.e.m. (n for Panels A, B, E, F = 6; for Panels C, D = 12).**

474 **Different letters represent statistically significant differences between groups ($p \leq 0.05$).**

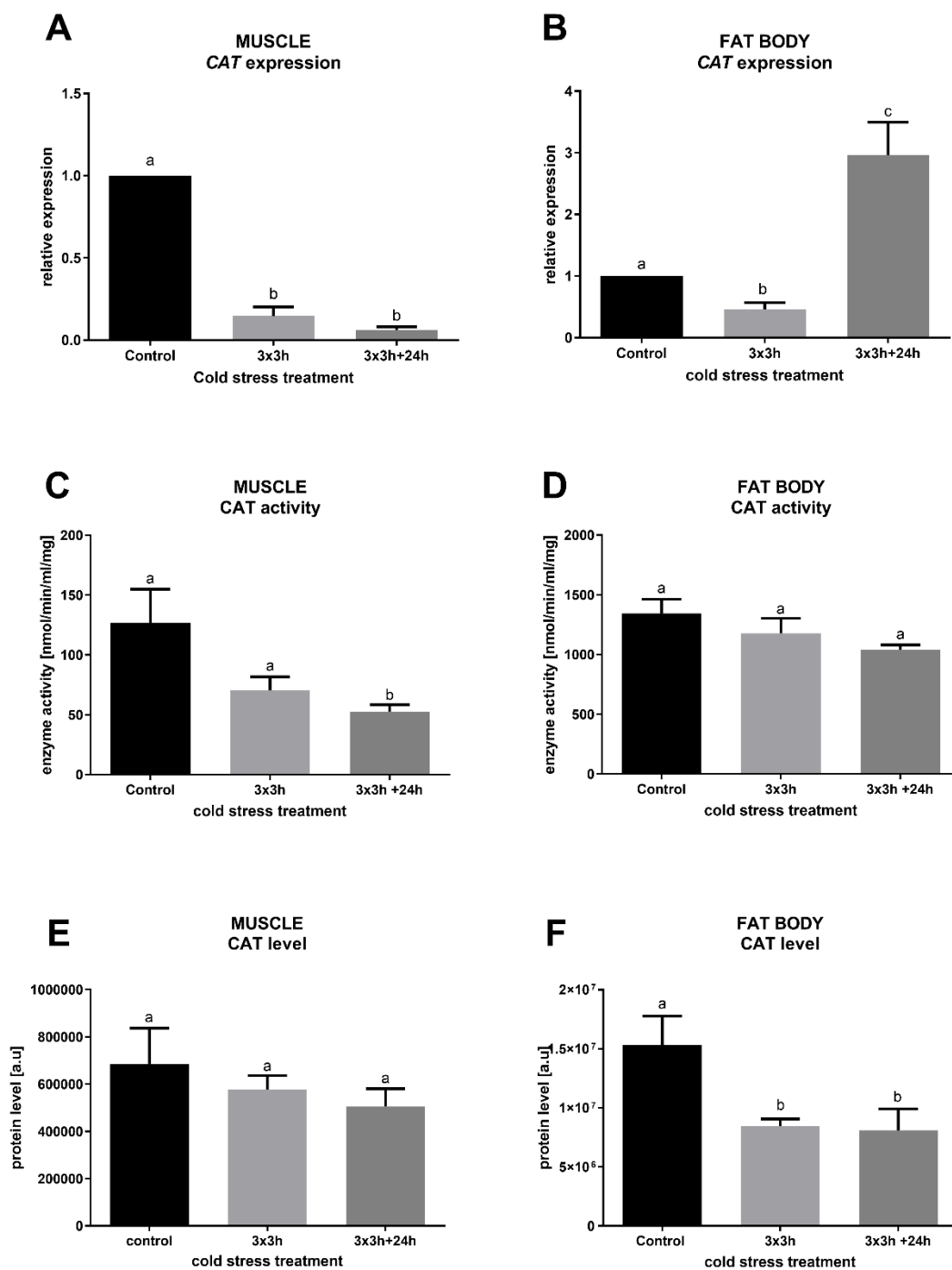
475 **Statistical significance was determined using one-way ANOVA with Tukey's multiple**

476

comparisons test.

477

478 A substantial decrease in *CAT* expression was observed in muscle tissues from both experimental
479 groups (Fig. 9A). *CAT* expression was downregulated by 6.8-fold in the 3 x 3 h of cold group ($p =$
480 0.0313) and 16.26-fold in the 3 x 3 h + 24 h recovery group ($p = 0.0313$). On the other hand, in the
481 fat body, such downregulation was observed only in the first experimental group (decrease by 2.15-
482 fold, $p = 0.0313$). In the 3 x 3 h + 24 h treatment group, a significant, 2.95-fold upregulation of
483 *CAT* was observed (Fig. 9B) ($p = 0.0313$). Although in most cases these changes are not
484 statistically significant, a similar trend of decreasing *CAT* activity was observed in both tissues. A
485 significant decrease was observed only in muscle tissue after 24 h of recovery ($p = 0.0194$) (Fig.
486 9C, D). After cold stress (3 x 3 h) and recovery (3 x 3 h + 24 h), the activity of the enzyme seemed
487 to decrease by 34.90% and 51.41%, respectively (Fig. 9C), in the muscles. Similarly, in the fat
488 body tissue, these values were 12.38% and 22.59% lower than those in the control group,
489 respectively (Fig. 9D). The changes in the protein level in muscles followed the same pattern as
490 that observed for the activity, showing a decrease over experimental procedures (Fig. 9E and Fig.
491 S3A). In the FB, a substantial decrease in the *CAT* protein level was also observed, that is, *CAT*
492 level was decreased by 44.78% and 47.32% in the 3 x 3 h and 3 x 3 h + 24 h groups, respectively
493 ($p = 0.0208$ and $p = 0.0386$, respectively) (Fig. 9F and Fig. S3B).



494

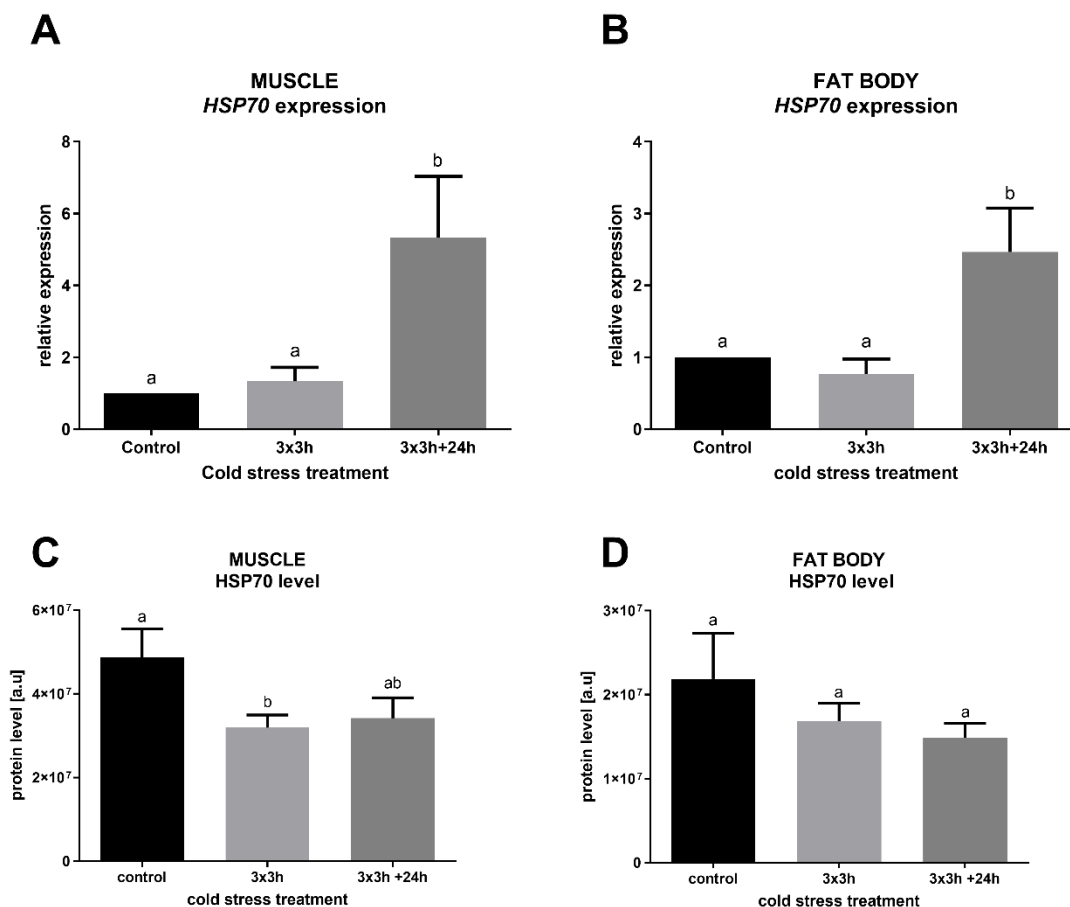
495 **Figure 9. The expression, activity and protein abundance of the CAT enzyme in the muscle**
 496 **(A, C and E) and fat body (B, D and F) tissues of *G. coquereliana* subjected to repeated cold**
 497 **stress (3 x 3 h) and after recovery (3 x 3 h + 24 h). The data are presented as the mean value ±**
 498 **s.e.m. (*n* for Panels A, B, E, F = 6; for Panels C, D = 12). Different letters represent statistically**

499 significant differences between groups ($p \leq 0.05$). Statistical significance was determined using
500 one-way ANOVA with Tukey's multiple comparisons test.

501 **3.2.4. The effect of cold stress on the expression of HSP70**

502 In muscle tissue, the expression of *HSP70* was affected by the treatments (ANOVA, $p =$
503 0.0142), and it was slightly, but not significantly, increased by 0.33-fold at ST 1 (Tukey's test, $p =$
504 0.9704), and it significantly increased by 4.33-fold at ST 2 (Tukey's test, $p = 0.0213$) (Fig. 10A).
505 At the protein level, after repeated stress, HSP70 abundance was significantly decreased by 34.54%
506 ($p = 0.0465$). After 24 h of recovery, the level of this protein increased by 7.05% compared to that
507 in the other treatment groups but was still approximately 29.93% lower than that in the control
508 insects (Fig. 10C and Fig. S4A).

509 In the fat body, the expression of *HSP70* after 3 x 3 h cold stress treatment remained at the same
510 level as in the control; however, after 3 x 3 h + 24 h treatment, it was significantly increased by
511 2.47-fold and 3.2-fold compared to the control and 3 x 3 h treatment groups, respectively (Tukey's
512 test, $p = 0.0339$ and $p = 0.0141$, respectively) (Fig. 10B). At the protein level, the abundance of
513 HSP70 exhibited a gradually decreasing trend, with 22.94% and 32.13% decreases in the 3 x 3 h
514 and 3 x 3 h + 24 h groups, respectively, compared to the control group; however, these decreases
515 were not statistically significant (Fig. 10D and Fig. S4B).



516
 517 **Figure 10. Gene (A, B) and protein expression (C, D) of HSP70 in the muscle (A, C) and fat**
 518 **body (B, D) tissues of *G. coquereliana* subjected to repeated cold stress (3 x 3 h) and after**
 519 **recovery (3 x 3 h + 24 h).** The data are presented as the mean value ± s.e.m. ($n = 6$). Different
 520 letters represent statistically significant differences between groups ($p \leq 0.05$). Statistical
 521 significance was determined using one-way ANOVA with Tukey's multiple comparisons test.
 522

523 4. Discussion

524 The response to cold stress in many insect species from temperate or subpolar regions has been
 525 well documented. However, little is known about the cold stress response and related mechanisms
 526 in tropical species, such as cockroaches. The tropical cockroach *G. coquereliana* was shown to be
 527 able to withstand repeated exposure to cold (e.g., 4°C) (Chowański et al., 2017; Chowański et al.,
 528 2015) or even partial freezing (Lubawy et al., 2019b) quite well, indicating that this insect is quite

529 well adapted to survive low temperatures. Freeze tolerance has evolved convergently among some
530 insect groups, including Blattaria (Sinclair et al., 2003), via the development of partial freeze
531 tolerance in species that are exposed to brief or repeated periods of cold (Sinclair et al., 2003). In
532 the present study, we characterize the bioenergetic parameters of mitochondria that were isolated
533 from fat body and muscle tissues as well as antioxidant capabilities in cockroaches subjected to a
534 fluctuating thermal regime. To date, only a few studies have been conducted on mitochondria
535 isolated from cold-stressed and/or acclimated insects (Chowański et al., 2017; Colinet et al., 2017;
536 Da-Re et al., 2014; Havird et al., 2020). Here, we analysed the RCR, as it is one of the most useful
537 measures of isolated mitochondria; the RCR reflects the adjustment of the respiration rate (electron
538 transport) to the actual demand for ATP, and it indicates the degree of mitochondrial uncoupling
539 (Brand and Nicholls, 2011). In *Drosophila melanogaster*, Colinet et al. (2017) showed that cold
540 stress and acclimation affect these parameters. Additionally, the efficiency of oxidative
541 phosphorylation was higher in cold-acclimated (CA) flies than that in their counterparts.
542 Additionally, the RCR was elevated in CA flies, which reflects higher mitochondrial oxidative
543 phosphorylation activity (State 3). Similarly, Havird et al. (2020) showed that in two montane
544 mayflies, namely, *Drunella coloradensis* and *Baetis* sp., state 3 (termed OXPHOS in the study),
545 coupling efficiency and maximal mitochondrial respiration were higher in insects that had
546 acclimated to low temperatures. On the other hand, measuring the respiration of fly *C. costata*
547 larvae showed that flies with freeze-sensitive (nondiapausing) phenotypes exhibited decreased
548 oxygen consumption rates, whereas the mitochondria of freeze-tolerant insects (diapausing, cold
549 acclimated) maintained respiratory capacity during cold stress (Štětina et al., 2020). Although these
550 experiments were conducted at higher temperatures, which showed that the cold *per se* causes a
551 decrease in oxygen consumption, the capacity to respire upon return to warm temperature is
552 increased in cold-acclimated insects. We observed that the RCR marginally decreased in both

553 tested tissues. This indicates that although single exposure to cold may uncouple respiration, as we
554 previously showed in *G. coquereliana* fat bodies (Chowański et al., 2017), the fluctuating
555 temperatures did not induce obvious mitochondrial dysfunction, although there is no absolute RCR
556 value that is diagnostic of dysfunctional mitochondria because RCR values are substrate- and tissue
557 dependent. Mitochondrial respiratory control is a complex function whose value depends on
558 numerous factors, and this complexity is its main strength: a change in almost any aspect of
559 oxidative phosphorylation change the RCR (Brand and Nicholls, 2011). This was also observed in
560 the trend towards an elevated ADP/O ratio in both tissues. In addition, we observed decreased
561 UCP4 activity in muscle mitochondria (especially at ST 2), which supported the value of this
562 parameter. UCPs, mitochondrial inner membrane proteins, are responsible for uncoupling electron
563 transport through the respiratory chain from ATP synthesis, leading to a decrease in the efficiency
564 of ATP synthesis (Sluse et al., 2006). Although we did not observe an increase in UCP activity as
565 previously observed during a single exposure (Chowański et al., 2017), which indicated
566 thermogenic activity, in the fat body, *UCP4* was upregulated after 24 h of recovery. This may be
567 related to the increased level of superoxide anion production that was observed in this tissue at ST
568 2. Since UCP activation decreases ROS production, UCPs are considered an antioxidant system
569 that counteracts oxidative damage in cells at the expense of the yield of oxidative phosphorylation
570 (Słocińska et al., 2011). The increase in O_2^- production after recovery may be a signal that results
571 in the higher expression of *UCP4* to produce the protein that is needed further to decrease the
572 potentially harmful levels of ROS, whose production is elevated after cold stress (Lalouette et al.,
573 2011). We have previously shown that in both tested tissues of *G. coquereliana*, UCPs decrease
574 the levels of superoxide anion (Slocinska et al., 2011; Slocinska et al., 2016). Additionally, it was
575 suggested by Alves-Bezerra et al. (2014) that in *Rhodnius prolixus*, UCP4 may participate in
576 antioxidative machinery and protect cells from the detrimental effects of ROS after cold stress.

577 Ulgherait et al. (2020) showed that *D. melanogaster per⁰¹* mutants, which exhibit constitutively
578 high expression of *Ucp4B* and *Ucp4C*, recover after cold shock much faster than control specimens.
579 This suggests that *per⁰¹* mutants generate more heat than control flies.

580 COX is an enzyme that is localized to the inner mitochondrial membrane (IMM) and is the final
581 electron acceptor in the respiratory chain (Kadenbach and Huttemann, 2015; McMullen and Storey,
582 2008b). Enzymes of the IMM have been shown to be repressed in insects during winter months. In
583 both freeze-avoiding and freeze-tolerant insects, a decrease in COX activity can be observed during
584 winter (Joanisse and Storey, 1994). In freeze-avoiding *E. scudderiana*, COX activity decreases by
585 approximately 30%; however, *COXI* mRNA transcript levels remain unchanged (McMullen and
586 Storey, 2008a). Similarly, Camus et al. (2017) did not observe any changes in *COX* in *Drosophila*
587 *simulans* between the test populations, even though these populations differed in cold tolerance.
588 We found that in *G. coquereliana*, COX activity differed in the different tissues. The decrease in
589 COX activity in muscles after cold stress was probably a consequence of the suppression of protein
590 synthesis to reduce the amounts of selected enzymes in mitochondria during cold exposure
591 (suppression of metabolic activity) or a consequence of the inhibition of enzyme activities, perhaps
592 by reversible controls (McMullen and Storey, 2008b). Interestingly, in our study, COX activity
593 was increased in the fat body after repeated cold stress. The fat body is a partial homologue of liver
594 and adipose tissue in vertebrates. In mammals, it is well established that cold acclimation and/or
595 chronic cold exposure causes an increase in COX activity in liver and adipose tissues (Terblanche
596 et al., 2000). In mammalian organs, COX also acts as an antioxidant enzyme, and it has a ‘pocket’
597 or ‘crypt’ in its active site in which radical intermediates are entrapped and retained until water is
598 formed (Chance et al., 1979). In our study, we did not observe an increase in reactive oxygen
599 species levels but rather a decrease in all measured parameters. Thus, COX may participate in

600 antioxidant defence in *G. coquereliana*; to our knowledge, this is one of the first reports to measure
601 tissue-specific COX activity in insects after cold stress, and this topic needs further investigation.

602 Typically, when insects are exposed to cold stress, an increase in antioxidant activity (SOD and
603 CAT) can be observed, which is the result of increased ROS production (Farahani et al., 2020; Gao
604 et al., 2013; Lalouette et al., 2011). However, this was not the case in this study, as neither treatment
605 induced oxidative stress (measured by superoxide production and lipid peroxidation), but the levels
606 of the measured parameters decreased immediately after cold stress. We also did not observe an
607 increase in the expression, abundance and activity of SOD and CAT, but rather, we observed stable
608 or decreasing levels depending on the treatment and/or enzyme (the only exception being *CAT*
609 expression in the FB at ST 2). In the response of the citrus red mite *Panonychus citri* to low-
610 temperature stress (5 and 10°C), SOD activity increased at the beginning of the stress response and
611 then decreased (Yang et al., 2010). This is consistent with the findings of An and Choi (2010),
612 where the early stage of exposure to acute changes in temperature resulted in oxidative stress that
613 was regulated by antioxidant enzymes, but continued stress caused by acute temperature exposure
614 resulted in decreased SOD activity. Our results could be explained by the fact that those two
615 enzymes are not the only enzymes in the whole antioxidant machinery. Mucci et al. (2021) showed
616 that in cold-stressed honey bees, H₂O₂ accumulation did not increase, and there was no increase in
617 catalase activity. However, the total antioxidant activity (TAS) of bees increased after cold stress,
618 suggesting a role for nonenzymatic antioxidant compounds in controlling ROS levels (Mucci et al.,
619 2021). The authors also determined that high H₂O₂ levels can be neutralized by peroxidases, as
620 their level significantly increases after cold stress (Mucci et al., 2021). As we did not determine the
621 activity of other antioxidant enzymes, it is possible that different mechanisms are responsible for
622 the decreased superoxide and lipid peroxidation levels. Additionally, other proteins (e.g., HSP90

623 and vitellogenin) have been associated with tolerance to cold stress (Ramirez et al., 2017). This
624 indicates that other antioxidant mechanisms that are induced by cold stress in cockroaches are
625 sufficient to maintain ROS at lower levels, allowing for the survival of insects. Insects subjected
626 to cold stress rely on two strategies to avoid oxidative stress: 1) an increase in the activity of
627 antioxidant enzymes, as is the case with chill-susceptible (in the case of these insects, also mild
628 uncoupling by UCP) and freeze-avoiding insects, or 2) a suppression of metabolism to reduce the
629 production of ROS and protect cells, as observed in freeze-tolerant species (Lubawy et al., 2022).
630 *G. coquereliana* seems to rely on the second mechanism, that is, lowering its metabolism. Since
631 this insect can survive partial freezing (Lubawy et al., 2019a), this might be an explanation.
632 However, this leaves the door open for research on this topic in tropical insects.

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

645 be proven, rates of anabolism and catabolism should be quantified using, for example,
646 calorespirometry or infrared gas analysis (Acar et al., 2001).

647 In most studied insects, HSPs are induced in response to cold stress (Jiang et al., 2012; Rinehart et
648 al., 2007). It's been shown that changes can be observed at both the gene and protein levels of
649 HSP70 during cold stress and recovery (Colinet et al., 2007; Colinet et al., 2010a; Goto and Kimura,
650 1998; Kimura et al., 1998). Our previous studies showed that after a single exposure to cold, new
651 isoforms of HSP70 protein are expressed (Chowanski et al., 2015), and the protein abundance
652 increases in muscle and fat body tissues (Chowanski et al., 2017). However, in this study, we
653 observed the opposite effect. The *HSP70* expression levels in both tissues seems to increase,
654 especially at ST2, while at the protein level, there was a decrease in HSP70 abundance (especially
655 in muscle at ST1). This might be explained by the time of observation. *HSPs* are known to be
656 highly upregulated shortly after stress and to be downregulated with time (Rinehart et al., 2007;
657 Rinehart et al., 2000). Even though it was statistically significant, the upregulation was rather low
658 (3-6-fold depending on tissue); hence, our observations are most likely reminiscent of higher levels
659 during cold stress that do not translate into higher protein levels.

660 In conclusion, this work shows that the bioenergetic parameters of mitochondria that were isolated
661 from the cockroach *G. coquereliana* do not change significantly after exposure to a FTR (Fig. S6)
662 compared to a single exposure to stress, which we previously studied (Chowanski et al., 2017);
663 these results indicate the presence of adaptive mechanisms in this tropical insect. Additionally,
664 during the FTR, the abundance of HSP70, together with the activity of antioxidant enzymes and
665 the levels of ROS, decrease. Interestingly, after 24 h of recovery, redox and energy balances,
666 especially in the fat body, are restored, which shows a substantial role of this tissue in intermediary
667 metabolism and energy management in insects as well as in response to repeated thermal stress.

668 Further research into the activity of enzyme that are related to major metabolic pathways and
669 metabolic changes in muscle and fat body tissues may allow us to elucidate and better understand
670 the mechanisms that are responsible for the cold stress response of *G. coquereliana*. Additionally,
671 understanding the physiological processes that occur under fluctuating temperatures is becoming
672 more desirable because, as noted by Marshall and Sinclair (2010), multiple exposures to cold
673 induce a trade-off between survival and future reproduction.

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677 **6. Competing interests**

678 The authors declare no competing interests.

679 **7. References**

680 References

681

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882

883 **Figures**

884 **Figure 1. Bioenergetic parameters of mitochondria isolated from the fat body tissue of *G.***
885 ***coquereliana*.** (A-B) Respiration was measured in the presence of 10 mM succinate and in the

886 absence (state 4 respiration) or presence (state 3 respiration) of 400 μ M ADP. C) RCR refers to the
887 respiratory control ratio. D) Changes in the ADP/O ratio of the fat body of *G. coquereliana*
888 cockroaches following repeated exposure to cold and recovery. The data are presented as the mean
889 value \pm s.e.m. ($n=7$). Different letters represent statistically significant differences between groups
890 ($p \leq 0.05$). Statistical significance was determined using one-way ANOVA with Tukey's multiple
891 comparisons test.

892 **Figure 2. Bioenergetic parameters of mitochondria isolated from the muscles of *G.***
893 ***coquereliana*.** (A-B) Respiration was measured in the presence of 10 mM pyruvate plus 10 mM
894 malate and in the absence (state 4 respiration) or presence (state 3 respiration) of 400 μ M ADP. C)
895 RCR refers to the respiratory control ratio. D) Changes in the ADP/O ratio of the muscles of *G.*
896 *coquereliana* cockroaches following repeated exposure to cold and recovery. The data are
897 presented as the mean value \pm s.e.m. ($n=7$). The same letters represent no statistically significant
898 difference between groups. Statistical significance was determined using one-way ANOVA with
899 Tukey's multiple comparisons test.

900 **Figure 3. Changes in the UCP activity of the muscle (A) and the fat body (B) of *G.***
901 ***coquereliana* cockroaches following cold exposure and recovery.** UCP activity was measured
902 in isolated mitochondria in the presence of substrate, palmitic acid (PA, an activator of UCP) and
903 GTP (an inhibitor of UCP). To exclude the activities of the ATP/ADP antiporter and ATP synthase,
904 carboxyatractyloside (1.5 μ M) and oligomycin (1 mg per 1 mg of mitochondrial protein) were
905 added to the incubation medium. Panels C and D present changes in *UCP* expression levels in the
906 muscles and the fat body of cockroaches, respectively. The data are presented as the mean value \pm
907 s.e.m. ($n=4$ for Panels A and B, $n=6$ for Panels C and D). Different letters represent statistically
908 significant differences between groups ($p \leq 0.05$). Statistical significance was determined using
909 one-way ANOVA with Tukey's multiple comparisons test.

910 **Figure 4. Activity of cytochrome c oxidase in the mitochondria of the muscles and the fat**
911 **body isolated from *G. coquereliana*.** The data are presented as the mean value \pm s.e.m. ($n=4$).
912 Different letters represent statistically significant differences in a given tissue between groups ($p \leq$
913 0.05). Statistical significance was determined using one-way ANOVA with Tukey's multiple
914 comparisons test.

915 **Figure 5. ATP levels in muscles and fat body tissues of *G. coquereliana* subjected to repeated**
916 **cold stress (3 x 3 h) and after recovery (3 x 3 h + 24 h).** The data are presented as the mean value
917 \pm s.e.m. ($n = 8$). Different letters represent statistically significant differences in a given tissue
918 between groups ($p \leq 0.05$). Statistical significance was determined using one-way ANOVA with
919 Tukey's multiple comparisons test.

920 **Figure 6.** Superoxide generation in mitochondria that were isolated from muscle and fat body
921 tissues of *G. coquereliana* after repeated cold stress (3 x 3 h) and recovery (3 x 3 h + 24 h). The
922 data are presented as the mean value \pm s.e.m. ($n=5-7$). Different letters represent statistically

923 significant differences in a given tissue between groups ($p \leq 0.05$). Statistical significance was
924 determined using one-way ANOVA with Tukey's multiple comparisons test.

925 **Figure 7. Lipid peroxidation level in muscle and fat body tissues of *G. coquereliana* after being**
926 **subjected to repeated cold stress (3 x 3 h) and after recovery (3 x 3 h + 24 h).** The data are
927 presented as the mean value \pm s.e.m. ($n=12$). Different letters represent statistically significant
928 differences in a given tissue between groups ($p \leq 0.05$). Statistical significance was determined
929 using the Kruskal–Wallis test with Dunn's multiple comparisons test.

930 **Figure 8. The expression (A and B), activity (C and D) and protein abundance (E and F) of**
931 **the SOD enzyme in the muscle (A, C and E) and fat body (B, D and F) tissues of *G.***
932 ***coquereliana* repeatedly exposed to cold stress (3 x 3 h) and recovery (3 x 3 h + 24 h).** The data
933 are presented as the mean value \pm s.e.m. (n for Panels A, B, E, F = 6; for Panels C, D = 12).
934 Different letters represent statistically significant differences between groups ($p \leq 0.05$). Statistical
935 significance was determined using one-way ANOVA with Tukey's multiple comparisons test.

936 **Figure 9. The expression, activity and protein abundance of the CAT enzyme in the muscle**
937 **(A, C and E) and fat body (B, D and F) tissues of *G. coquereliana* subjected to repeated cold**
938 **stress (3 x 3 h) and after recovery (3 x 3 h + 24 h).** The data are presented as the mean value \pm
939 s.e.m. (n for Panels A, B, E, F = 6; for Panels C, D = 12). Different letters represent statistically
940 significant differences between groups ($p \leq 0.05$). Statistical significance was determined using
941 one-way ANOVA with Tukey's multiple comparisons test.

942 **Figure 10. Gene (A, B) and protein expression (C, D) of HSP70 in the muscle (A, C) and fat**
943 **body (B, D) tissues of *G. coquereliana* subjected to repeated cold stress (3 x 3 h) and after**
944 **recovery (3 x 3 h + 24 h).** The data are presented as the mean value \pm s.e.m. ($n = 6$). Different
945 letters represent statistically significant differences between groups ($p \leq 0.05$). Statistical
946 significance was determined using one-way ANOVA with Tukey's multiple comparisons test.

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