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**TITLE: *Thermal stress causes DNA damage and mortality in a tropical insect***

**RUNNING TITLE: Thermal stress causes DNA damage & mortality in insect**

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**Abbreviations:** tail length (TL), percentage of total DNA in the tail (%DNAT), olive tail moment (OTM) and percentage of cells with visible comets (%COM), single cell gel electrophoresis assay (SCGE), supercooling point (SCP), reactive oxygen species (ROS), anticoagulant buffer (ACB), heat shock proteins (HSPs), aquaporin (AQP), total body water (TBW), lower lethal temperature (LLT),

**Key words:** *Gromphadorhina coquereliana*, cockroach, freezing, heat stress, Comet assay, DNA damage

**Summary:**

- Tropical cockroach *Gromphadorinha coquereliana* survives temperatures below 0°C
- *G. coquereliana* can be considered as moderately freezing-tolerant species
- Temperature stress causes substantial DNA damage in haemocytes of the cockroach
- This cockroach possesses an efficient DNA repair system

**Abstract:**

Cold tolerance is considered an important factor determining geographic distribution of insects. We've previously shown that despite tropical origin, cockroach *Gromphadorinha*

*coquereliana* is capable of surviving exposures to cold. However, freezing tolerance of this species had not yet been examined. Low temperature is known to alter membranes integrity in insects but whether chilling or freezing compromises DNA integrity remains a matter of speculation. In the present study, we subjected the *G. coquereliana* adults to freezing to determine their supercooling point (SCP) and evaluated whether the cockroaches were capable of surviving partial and complete freezing. Next, we conducted single cell gel electrophoresis assay (SCGE) to determine whether heat, cold and freezing altered haemocytes DNA integrity. The SCP of this species was high and around -4.76°C, which is within typical range of freezing-tolerant species. Most cockroaches survived one day after partial ice formation (20% mortality), but died progressively in the next few days after cold stress (70% mortality after 4 days). One day after complete freezing, most insects died (70% mortality), and after 4 days, 90% of them had succumbed. The SCGE assays showed substantial level of DNA damage in haemocytes. When cockroaches were heat-stressed, the level of DNA damage was similar to that observed in the freezing treatment; though all heat-stressed insects survived. The study shows that *G. coquereliana* can surprisingly be considered as moderately freezing-tolerant species, and that extreme low temperature stress can affect DNA integrity, suggesting that this cockroach may possess an efficient DNA repair system.

## 1. Introduction

The majority of insect species survive, thrive and remain active within a limited range of temperatures (Chown and Nicolson, 2004). This thermal range depends on species geographical origins, with tropical species generally showing narrower thermal range than temperate ones (Kellermann et al., 2009). Cold tolerance is considered as an important factor determining geographic distribution of insect species (Addo-Bediako et al., 2000). In the course of evolution, insects exposed to low temperature developed a number of adaptations to survive suboptimal thermal conditions (Lee Jr, 2010; Wharton, 2007). To resist when temperature falls below the freezing point, insects usually show two main strategies: freezing tolerance vs. freezing intolerance (Lee, 1991; Sinclair et al., 2003b; Sømme, 1999). When freezing is tolerated, it is strictly limited to extracellular compartments, as intracellular freezing is lethal to most animals, with some exceptions (e.g. some nematodes) (Block, 2003; Storey and Storey, 1989). Freezing intolerance is the strategy found in a large majority of

arthropods (Block, 1990; Lee and Costanzo, 1998). To survive cold, freezing-intolerant species rely on mechanisms by which they increase their cold-tolerance and their capacity to remain unfrozen by supercooling (Sformo et al., 2010). The temperature at which ice forms is termed the supercooling point (SCP), as it denotes the ultimate limit of supercooling (Lee Jr, 2010). The SCP is determined by detecting the latent heat of crystallization (the exotherm) released as body fluids start to freeze. The SCP obviously represents the lower lethal temperature (LLT) for freezing intolerant insects; however, many species die at temperatures well above SCP due to chilling injuries (Bale, 2002; Overgaard and MacMillan, 2017). Hence, the true ecological value of the SCP has been largely debated (Ditrich, 2018; Renault et al., 2002). In spite of this, often studies exploring cold tolerance of poorly described species start with SCP measurements as it provides an anchor point about which the cold tolerance strategy can be determined (Sinclair et al., 2015).

Responses to cold have been well documented in many species from temperate (Chen et al., 1987; Czajka and Lee, 1990; Teets et al., 2012) and subarctic regions (Clark et al., 2009; Clark and Worland, 2008; Montiel et al., 1998). Cold adaptation and thermal limits of populations and species are supposed to be selected to match temperatures that characterize their geographic ranges and origins (Angilletta Jr and Angilletta, 2009; Ditrich et al., 2018; Sunday et al., 2012). It results that temperate populations usually better cope with low temperature stress than their tropical counterparts, as reported in flesh flies for instance (Chen et al., 1990). Likewise, *Drosophila* species from tropical origins are often much less cold tolerant than species found in temperate areas (Gibert et al., 2001; Goto and Kimura, 1998; Kellermann et al., 2012; Mensch et al., 2017; Olsson et al., 2016). However, whether species adapted to tropical climates are capable of tolerating cold stress, and by which physiological mechanisms they can do so, remains a poorly explored question. Bale described tropical insects as 'opportunistic survivors' regarding cold stress (Bale, 1993; Bale, 1996) and with climate changes occurring nowadays, knowledge about thermal tolerances of tropical insects is valuable due to potential expansions of invasive species as its result (Rodriguez-Castaneda et al., 2017).

Although most cockroach species are tropical, some species are adapted to extreme environments, such as dry desert or cold climates (Bell et al., 2007; Mullins, 2015). The diversity of habitats in which cockroaches are found reflects their great adaptability to cope with environmental stressors. Various cold hardiness strategies have been reported in

cockroaches. For instance, freeze avoidance is realized by microhabitat selection in *Periplaneta japonica* (Tanaka, 2002). Some cockroach species acquire cold hardiness by gradual acclimation, as reported in *Blatta orientalis* (Lepatourel, 1993). Freeze tolerance has also been reported in some species, such as in *Cryptocercus punctulatus* or *Celatoblatta quinquemaculata*, that utilize ice nucleating proteins and cryoprotectants (glycerol and trehalose) to allow freezing to be tolerated (Hamilton et al., 1985; Wharton, 2011; Wharton et al., 2009; Worland et al., 2004).

In insects, cold exposure at temperatures above SCP can induce chilling (non-freezing) injuries that develop as a result from complex physiological alterations such as loss of ion and water homeostasis, which participate to the disruption of neuromuscular functions, leading to chill coma and ultimately death (Overgaard and MacMillan, 2017). Kostal et al. (2006) previously showed that chilling injury resulted in the disturbance of ion homeostasis in the coxal muscle of tropical cockroach *Nauphoeta cinerea*. We have previously shown in another tropical cockroach from Madagascar, *Gromphadorinha coquereliana*, that they were surprisingly capable of surviving short term as well as repeated exposures to low temperatures (Chowanski et al., 2017; Chowanski et al., 2015). In the wild, this species is not supposed to be often exposed to cold as temperature can only occasionally drop to 4 °C for a few hours in Madagascar (Chowanski et al., 2015). In spite of this, we previously found that chilling triggered physiological response, such as heat shock proteins (HSPs) and aquaporins (AQPs) induction or metabolic responses, such as an increase in quantity of total protein in fat body, higher level of polyols and glucose in hemolymph and changes in mitochondrial respiration activity (Chowanski et al., 2017; Chowanski et al., 2015). The rather high chilling tolerance of *G. coquereliana* is rather surprising considering the tropical origin of this species (Chowanski et al., 2017).

Cockroaches are known to be resilient to many kinds of stresses such as hypoxia (Harrison et al., 2016), hypercapnia (Snyder et al., 1980), heat (McCue and De Los Santos, 2013), starvation (Duarte et al., 2015), or xenobiotics (Pietri et al., 2018). Many species exhibit discontinuous respiration and which is supposed to reduce water loss, improving survival to food and water restriction (Schimpf et al., 2012; Schimpf et al., 2009). Urates play a central role in cockroach physiology. Typically, fat body contains urocyte cells that contain stored urates (Cochran, 1985; Park et al., 2013). Many studies have confirmed that stored urates serve as ion sink, allowing for sequestration/release of hemolymph ions as a mechanism for

122 maintenance of ion homeostasis (Hyatt and Marshall, 1985a; Hyatt and Marshall, 1985b). This  
123 adaptation may be of particular relevance for cold tolerance as maintenance of ion  
124 homeostasis across membranes is key element of chilling tolerance (reviewed by Overgaard  
125 and MacMillan (2017).

126 In addition to the alteration of nuclear and cell membranes (Lee Jr, 2010; Quinn, 1989;  
127 Ramløv, 2000), DNA integrity may also be compromised by chilling and freezing. Only one  
128 study in *Musca domestica* have reported that chilling could cause nuclear anomalies (e.g.  
129 micronucleus) and chromosomal aberrations (e.g. stickiness, fragmentation or constrictions)  
130 (Mishra and Tewari, 2014). Upregulation of several transcripts in cold-stressed bees (e.g.  
131 myofilin isoform b, sestrin-like and DNA damage-binding proteins) suggests that insects may  
132 experience DNA damage, potentially caused by increased level of reactive oxygen species  
133 (ROS) (Torson et al., 2017). Whether or not cold and freezing stress can damage DNA has not  
134 yet been examined, other than at chromosomal level.

135 In humans and in other vertebrates, *in vivo* studies commonly use lymphocytes as the  
136 main target cells for measuring DNA damage because it is a non-invasive method (Azqueta  
137 and Collins, 2013; de Lapuente et al., 2015; Odongo et al., 2019). Invertebrates have  
138 haemocytes in the haemolymph that have the same role as lymphocytes and that can be used  
139 to assess DNA damage in cells (Adamski et al., 2019; Augustyniak et al., 2016; de Lapuente et  
140 al., 2015; Gaivao and Sierra, 2014). *Gromphadorinha coquereliana* is a rather large insect that  
141 possess high number of circulating haemocytes. This allows cells to be extracted and  
142 measured on single individuals, which is an advantage compared to small insects like  
143 *Drosophila melanogaster*, that requires hemolymph pooling from many specimens and mixing  
144 the material (Carmona et al., 2015). This model of tropical origin, is then particularly  
145 appropriate to address whether thermal stresses (heat, cold and freezing) can affect DNA  
146 integrity of circulating cells.

147 As mentioned before, urates metabolism plays central role in cockroach physiology (Park  
148 et al., 2013). Stored urates (in urocytes) is a particular adaption of cockroaches that  
149 contributes to ionic balance and osmoregulation by ion exchanges in tissue fluids (Mullins,  
150 2015; Mullins and Cochran, 1974). Since maintenance of metal ions homeostatis is directly  
151 linked to chilling tolerance of insects (Grumiaux et al., 2019; MacMillan et al., 2016; Overgaard  
152 and MacMillan, 2017) it is conceivable that this particularity may contribute to the  
153 unsuspected high chilling tolerance of *G. coquereliana* (Chowanski et al., 2017; Chowanski et

al., 2015). However, freezing resistance of this species had not yet been examined. In the present study, we first subjected the adult males *G. coquereliana* to freezing temperature to determine their supercooling point (SCP) and we evaluated whether the cockroaches were capable of surviving partial and complete freezing. Next, we conducted single cell gel electrophoresis assay (SCGE) to determine whether heat, cold and freezing stress altered DNA integrity of haemocytes.

## **2. Materials and Methods**

### **2.1. Insect rearing**

Cockroaches (*Gromphadorhina coquereliana*) were reared under laboratory conditions in continuous colony at 28 °C and approximately 65% relative humidity under a 12 h light/12 h dark cycle in Department of Animal Physiology and Development, AMU in Poznań. Food (lettuce, carrots, and powdered milk) and water were provided *ad libitum* as described previously (Slocinska et al., 2013). Only adult male individuals of approximately 5.9 cm ( $\pm$  0.39 cm) in size and a weight of 5.5 g ( $\pm$  0.48 g) were used for experiments.

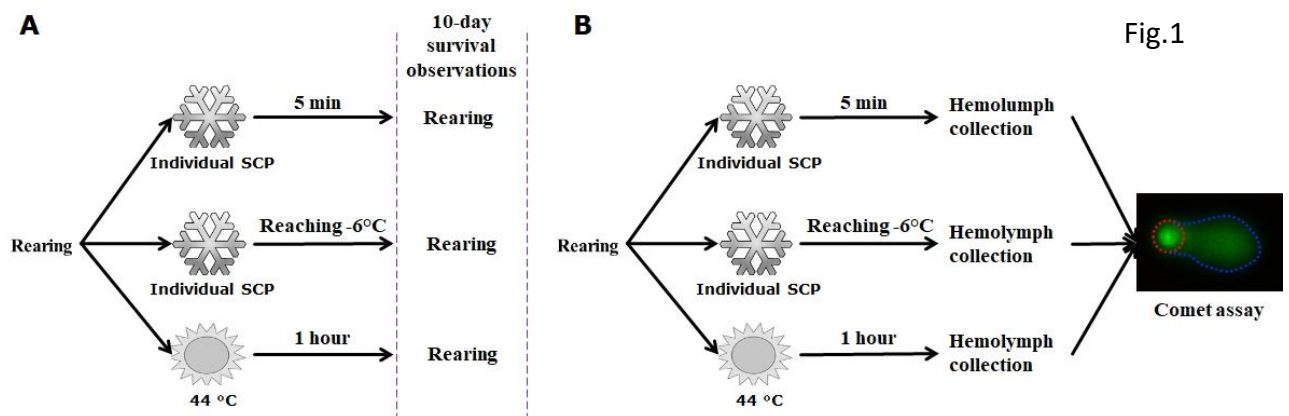
### **2.2. Determination of supercooling point (SCP)**

To determine SCP, insects were placed individually in 50 ml falcon tubes, which were submerged in cryostat bath (Polystat CC3, Huber Kältemaschinenbau AG, Germany) filled with heat transfer fluid (Thermofluid SilOil, Huber, Germany). The temperature of the bath was slowly reduced at a 0.5 °C/min rate to reach target temperature of -30 °C. To monitor the temperature of the insects, a K-type thermocouple was placed in the middle of the dorsal side of the cockroach, touching the cuticle, secured with Parafilm® and connected to temperature data logger Testo 175T3 (Testo SE & Co., Germany). The temperature of the insects was recorded every 10 seconds. The SCP was defined as the temperature at the onset of the freezing exotherm produced by the latent heat. We used 24 insects to determine SCP.

### **2.3. Thermal treatments**

Insects were subjected to low temperature stress (both cold and freezing), as well as heat stress (Fig. 1). In the first low temperature treatment (denoted as COLD), each insect was

brought to its SCP and left in the water bath with ongoing decrease in temperature for only 5 min after reaching it, which resulted in incomplete freezing, with small proportion of the total body water (TBW) frozen. In the second low temperature treatment (denoted as FREEZE), insects were brought to their SCP and left in the water bath until the temperature of insect dropped again and reached -6 °C (on average it took 20 min) after the exotherm, which resulted in freezing of most of the TBW of the specimen. For heat stress (denoted as HEAT), insects were placed in 1 L glass bottle submerged in water bath set to 44 °C (VariostatCC, Huber Kältemaschinenbau AG, Germany) for 1 h. We selected these experimental conditions, based on preliminary tests that showed that insects were deeply stressed (hyperventilating and unable to stand on legs), but still alive after 1 h. Each time, three insects were placed in the bottle at the same time. The bottle was secured with sponge plug in order to prevent the insects from escaping and allowing for the circulation of air. Temperature inside bottles was precisely adjusted with K-type thermocouples placed inside an immersed empty bottle. Ten different individuals were used for each of the three thermal treatments ( $n = 10$ ). Hemolymph was collected one hour after each treatment.



**Fig. 1. Scheme of the experimental designs for the survival experiments (A) and comet assay (B)**

## 2.4. Survival

After each thermal treatment, 10 individuals were placed in a breeding room in plastic boxes (15 x 30 x 20 cm) with carrots for food to test for survival after stress exposure. Mortality was recorded at 1 h after stress and each day after, for a period of 10 days. The insects were



considered dead when they did not react to the pinch of the legs and antenna using forceps. An untreated control group of 10 insects was also monitored during same period.

## **2.5. Assessment of DNA integrity of haemocytes**

Circulating haemocytes were isolated by collecting hemolymph from single treated individuals. To do so, insects were anaesthetized by submersion, as previously described (Chowanski et al., 2017). Because hemolymph coagulates rapidly, after anesthesia, insects were injected with 300 µL of anticoagulant buffer [ACB] (69 mM KCl, 27 mM NaCl, 2 mM NaHCO<sub>3</sub>, 30 mM sodium citrate, 26 mM citric acid and 10 mM EDTA, pH 7.0, Sigma-Aldrich, St. Louis, USA) (Chowanski et al., 2017; Chowanski et al., 2015). The ACB was injected under the last pair of legs using a Hamilton syringe (Hamilton Co., Reno, USA) and then insects were left for 5 min to allow the ACB to spread throughout the insect body. To avoid any UV-related DNA damage of isolated cells, all the following steps were performed in a light-protected room. After injection of the ACB, the last right leg was cut off at the coxa and 100 µL of hemolymph was collected in a 1.5 mL tube filled with 100 µL of ACB. The samples were centrifuged at 1000g for 10 min at 4 °C. The supernatant was discarded and the haemocytes were resuspended in 100 µL of ACB. Cells were diluted 100x with ACB to obtain ~2.5 x 10<sup>5</sup> cells/mL in all samples. To assess DNA integrity of isolated haemocytes, Comet SCGE assay kit (ENZO Life Sciences, Inc., New York, USA) was used according to manufacturer's instructions. Briefly, the cells were combined with molten LMAgarose in 1:10 (v:v) ratio and immediately pipetted onto microscope slides. After gelling of the agarose, the slides were placed in pre-chilled Lysis solution for 60 min. Next the slides were immersed for 30 min in Alkaline Solution (300 mM NaOH, 1 mM EDTA, pH >13) and then washed twice in 1xTBE buffer (80mM Tris Base, 89mM boric acid, 3.2 mM EDTA) for 2 min. Slides were then placed flat onto a gel tray and align equidistant from the electrodes. The voltage was set to 1 volt per cm (measured electrode to electrode) and applied for 10 min. After electrophoresis samples were dipped in 70% ethanol for 5 minutes and dried in an incubator set to 37 °C. Comets were stained with 10x CYGREEN® Dye for 30 min and visualized by Olympus BX41 epifluorescence microscope (Olympus, Tokio, Japan, FITC filter - excitation/emission 489/515 nm) equipped with LEICA DFC450 C camera (Leica, Wetzlar, Germany). In order to provide a positive control for each step in the comet assay, one slide with cells that had been treated with H<sub>2</sub>O<sub>2</sub> was prepared for every electrophoretic run. The cells, which were isolated from randomly selected control animals,

were treated with H<sub>2</sub>O<sub>2</sub> (100 µM, Sigma-Aldrich, St. Louis, USA) for 10 min at 4 °C, and after which they were tested for DNA damage under the same conditions as described above. For each thermal treatment, 5-6 different treated animals were used ( $n = 5$  or  $6$ ), each produced one slide of haemocytes to analyze. In addition, five untreated (unstressed) insects were also used to assess DNA integrity of their haemocytes, and these controls were processed exactly as the treated specimens. At least 50 nuclei from 10 randomly captured images were analyzed per slide (i.e. 10 images/slide and 5-6 slides/treatment).

## **2.6. Image analyses**

All images were analyzed with CASP Lab software (Konca et al., 2003). Tail DNA is the most commonly used parameter to assess DNA integrity, but the other metrics are also frequently used (Collins et al., 1997; Kumaravel and Jha, 2006). In the present study, DNA damage was evaluated by: (i) comet tail length (TL) which shows the length of DNA migration; (ii) percentage of total DNA in the tail (%DNAT), defined as the amount of DNA that has migrated out of the nucleus expressed as the percentage of total cellular DNA content; (iii) Olive tail moment (OTM), which gives an estimation of the relative proportion of DNA at different regions of the tail, and (iv) percentage of cells with visible comets (%COM). OTM is the distance between the center of mass of the tail and the center of mass of the head, in micrometers, multiplied by the percentage of DNA in the tail. OTM is considered the most sensitive comet parameter as both the quality and quantity of DNA damage are taken into account (Dhawan et al., 2009).

## **2.7. Statistical analysis**

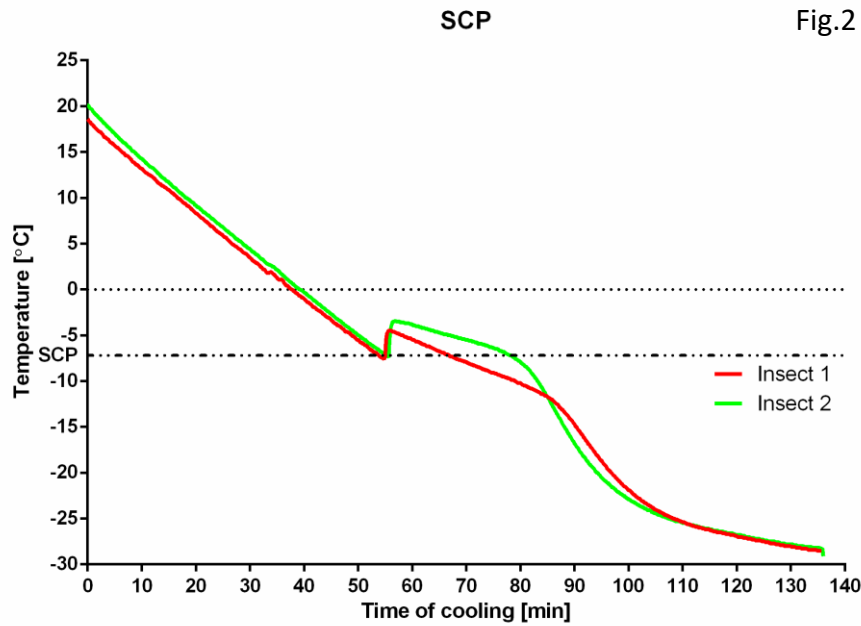
For survival analyses, Mantel-Cox test with mortality at 10 days was used. For all tests,  $p$ -values lower than 0.05 were considered as statistically significant. The data are presented as mean  $\pm$ SEM. For DNA damage comparisons, because multiple pictures (10) were taken from the same treated individuals, the identity of each insect (or each slide) was incorporated in the model as a random variable to account for multiple measures. In addition, since characteristics of all cells analyzed within a picture might be dependent on the capture settings of each picture, the picture identity (nested within each insect identity) was also included as random variable in the model. Therefore, mixed effects generalized model (GLMM) was applied for each tested parameter using the function '*lmer*' in the '*lme4*' package

for R. When the explanatory variable (i.e, treatments) was significant, we conducted Holm-adjusted Tukey's pairwise comparison tests using function 'glht' in the package 'multcomp' in R (Venables et al., 2018).

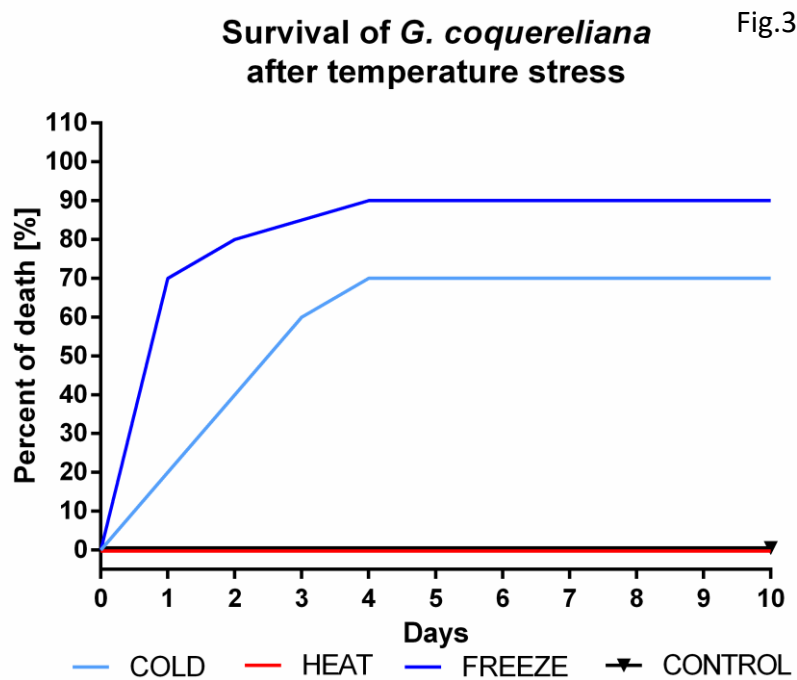
### 3. Results

#### 3.1. Determination of the SCP and survival of stress treatments

Supercooling points of adult males varied from -7.6 °C to -1.9 °C with an average of  $-4.76 \pm 1.60$  °C ( $n = 24$ ) and the mean time to complete freezing was  $18.08 \pm 11.38$  min. The SCPs of individuals after COLD and FREEZE treatments were  $-4.78 \pm 1.00$  °C (min. -6.00 °C, max. -2.80 °C) and  $-4.26 \pm 1.56$  °C (min. -6.30 °C, max. -2.30 °C), respectively. Fig. 2. shows typical cooling curves of *G. coquereliana* with exotherms. 80% of insects survived 24 h after the COLD treatment. Over half of the insects died during the first 3–4 days after COLD treatment (Fig. 3) and only 30% of them have survived the 10 days, not showing any visual signs of being affected by the COLD treatment (i.e., partial freezing). The survival curve for COLD treatment was statistically different from CONTROL group ( $\chi^2 = 10.59$ ;  $p = 0.001$ ). 30% of insects from the FREEZE group have survived 24 h after complete freezing. However, they showed clear signs of injuries, such as inability to walk, feeble movements of appendices, and weak responses to stimulations. Only 10% of insects from this group survived 10 days after freeze treatment. The survival curve of the FREEZE treatment was statistically different from CONTROL group ( $\chi^2 = 17.20$ ;  $p < 0.001$ ). During the HEAT treatment, all insects showed significant signs of stress: uncoordinated movement, increased abdominal ventilation frequency and defecation. In spite of this, all cockroaches survived HEAT treatment for 10 days, showing no statistical differences compared to control ( $p > 0.05$ ).



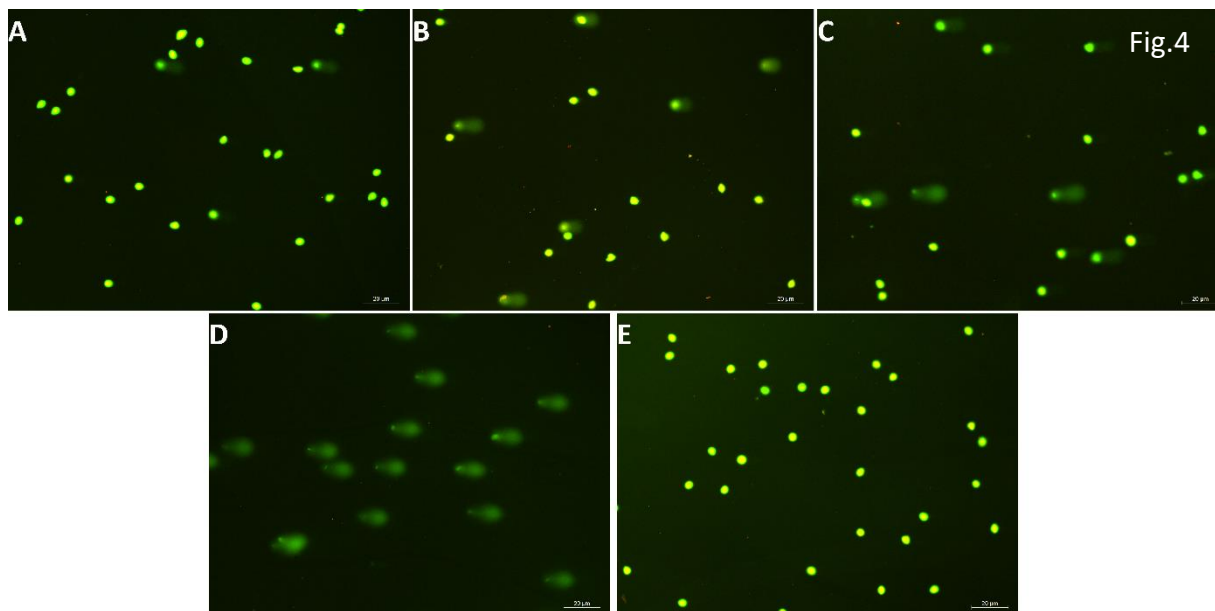
**Fig. 2. Exemplary supercooling curves for two individual adult males of *G. coquereliana*.** The temperature ramp was set at a -0,5 °C/min rate to reach target temperature of -30 °C.



**Fig. 3. Survival curves of adult males of *G. coquereliana* after COLD, FREEZE and HEAT treatment over 10 days period.** For survival analyses, Mantel-Cox test with mortality at 10 days was used. Each group consisted of 10 individuals.

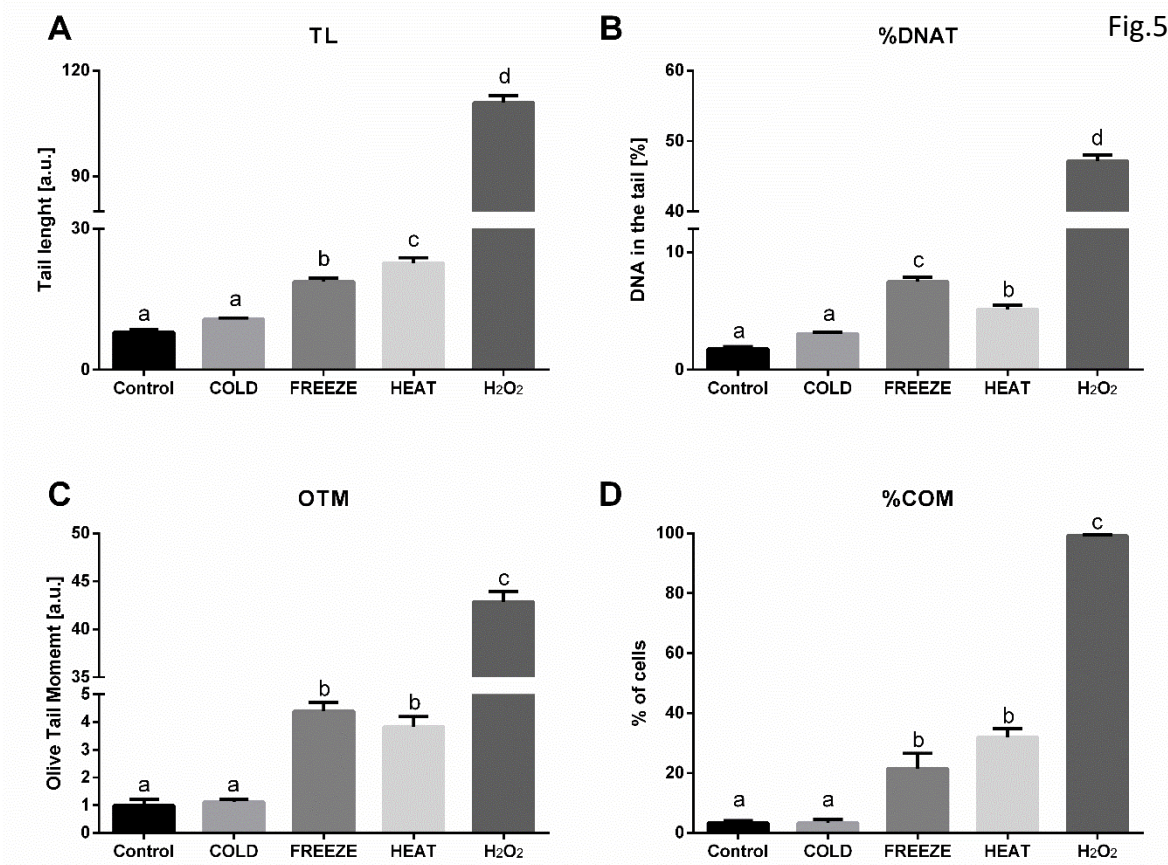
### 3.2. DNA damage

Figure 4 shows the effects of treatments on the isolated haemocytes and Figure 5 the results of all the DNA damage metrics according to the three thermal treatments, the untreated control and the positive  $\text{H}_2\text{O}_2$ -treated control. The mean values differed significantly according to treatments in all tested parameters (TL:  $\chi^2 = 6496.2$ ,  $df = 4$ ,  $p < 0.001$ ; %DNAT:  $\chi^2 = 6968.5$ ,  $df = 4$ ,  $p < 0.001$ , OTM:  $\chi^2 = 5859.6$ ,  $df = 4$ ,  $p < 0.001$  and %COM:  $\chi^2 = 6441.4$ ,  $df = 4$ ,  $p < 0.001$ ). These differences were mainly driven by  $\text{H}_2\text{O}_2$ -treated samples that, as expected, showed substantial DNA damages. Pairwise multiple comparisons were used to discriminate significance of the different treatments, as indicated by different letters in Figure 5. For all tested parameters, comparisons showed that DNA damages were much superior in  $\text{H}_2\text{O}_2$  samples than in the other treatments (Tukey tests,  $p < 0.001$ ). Values of FREEZE or HEAT treatments were lower than in  $\text{H}_2\text{O}_2$ , but clearly superior than control (Tukey tests,  $p < 0.001$ ). Finally, we found no clear indication, in any parameter, that COLD treatment induced DNA damage, as indicated by lack of significant difference with control (Tukey tests,  $p < 0.001$ ).



**Fig. 4. Representative images of effects of temperature stress on DNA damage in haemocytes isolated from adult males of *G. coquereliana* after A) COLD, B) FREEZE and C) HEAT treatments. Picture D) represents positive control where cells were treated with  $\text{H}_2\text{O}_2$  at 100  $\mu\text{M}$  concentration and E) insects from negative control (see materials and methods for**

details). Scale represents 20  $\mu\text{m}$ . To visualize the DNA the preparations were stained with CYGREEN® Dye.



**Fig. 5. Effects of temperature stress on DNA damage in haemocytes of adult males of *G. coquereliana*.** A) Length of the comet tail (TL), B) Percentage of DNA in the comet tail (%DNAT), C) Olive tail moment (OTM) and D) Percentage of cells with visible comets (%COM) of all experimental animal groups. Mixed effects generalized model (GLMM) was applied for each tested parameter and Holm-adjusted Tukey's pairwise comparison test using R. All data are expressed as mean  $\pm$ SEM. Different letters on the bars indicate significant differences between means.

## 4. Discussion

In this report we show that, although the cockroach *G. coquereliana* is a tropical species endemic to Madagascar (Beccaloni, 2014) it can be considered as moderately freezing-tolerant one. We also show for the first time that cold stress does not damage substantially the DNA whereas extreme low temperature stress can affect DNA integrity in haemocytes of insects.

We found that the cockroaches were able to survive after they experienced the onset of freezing. However, when the body temperature reached  $-6^{\circ}\text{C}$  after reaching SCP (i.e. complete freezing), the mortality increased to 90%. The SCP data obtained here indicate that spontaneous freezing (SCP) occurred in *G. coquereliana* at temperatures a few degrees under zero ( $-4.7 \pm 1.6^{\circ}\text{C}$ ). This is within the typical range of freezing tolerant cockroach species, like the alpine cockroach *Celatoblatta quinquemaculata* or *Periplaneta japonica* that avoid supercooling and promote freezing at relatively high subzero temperatures with ice nucleators and cryoprotectants (Sinclair, 1997; Tanaka and Tanaka, 1997; Wharton et al., 2009; Worland et al., 1997). On contrary, freezing avoiding insects generally exhibit deep supercooling ability, with SCP values often in the range of  $-15$  to  $-25^{\circ}\text{C}$  or lower (Danks, 2004; Vernon and Vannier, 2002). Although the SCP profile of *G. coquereliana* looks exactly as those reported in freezing tolerant *C. quinquemaculata* (Worland et al., 1997; Worland et al., 2004), it would be surprising that *G. coquereliana* has developed (or inherited) physiological adaptations to survive freezing. The Blattodea is phylogenetically old order of insects and cockroaches are likely one of the most primitive of living neopteran insects. Most of the species from this order inhabit in temperate or tropical zones, however according to Sinclair et al. (2003a) they are mostly freezing tolerant insects (Sinclair et al., 2003a). Hence cold adaption may be an ancestral heritage within this order. Alternatively, it could be explained by the modification of pre-adapted pathways (exaptation, sensu Gould and Vrba (1982). Predictions of this hypothesis include substantial overlap between freezing and desiccation tolerance in insects, so that physiological adaptations to desiccation stress promote cross-tolerance to freezing (Hayward et al., 2007). As reported in another species, *C. quinquemaculata* (Sinclair, 1997), *G. coquereliana* survived initiation of ice formation but die when body temperature was further reduced after onset of freezing. So it can be classified as moderately freezing tolerant (referring to partial freezing tolerance) (Sinclair, 1999). Several other insects have been

classified as moderately freeze tolerant including the subantarctic beetle, *Hydromedion sparsutum*, from South Georgia which freezes at c.a.  $-2.5^{\circ}\text{C}$  and survives frozen to c.a.  $-8^{\circ}\text{C}$  (Worland and Block, 2003). Partial freeze tolerance may be an evolutionary route to freeze tolerance, for instance in species that are exposed to brief periods in the cold (e.g. the variable habitats of the southern hemisphere or tropical high mountains (Sinclair et al., 2003a). The significance of SCP has been questioned for tropical species that actually rarely experience subzero temperatures (Renault et al., 2002). As mentioned before, mechanisms of cold tolerance in tropical species may be unrelated to cold adaption *per se* and may be rather linked to some other native characteristics of the species, such as desiccation- mechanisms. Indeed, freeze and desiccation tolerance share many characteristics and the biochemical and cellular mechanisms for freeze tolerance have been suggested to evolve via cross-tolerance for desiccation (Toxopeus and Sinclair, 2018).

Freezing is associated with osmotic dehydration of cells and loss of extracellular ion balance linked to a complex of deleterious alterations such as depolarization of membranes and altered fluidity (Muldrew et al., 2004; Overgaard and MacMillan, 2017; Overgaard et al., 2005). In *M. domestica*, cold stress was reported to lead to increase in chromosome aberrations and micronucleus frequency occurrence (Mishra and Tewari, 2014). Whether cold and freezing stress can damage DNA had not yet been examined in any insect models.

When cockroaches were heat-stressed the observed DNA damages were similar to those from FREEZE treatment. However, the survival of the insects from this group was 100%, even though there were evident signs of sublethal effects (i.e. increased hyperventilation). Both TL and OTM were superior in both treatments compared to control. OTM incorporates quantitative and qualitative measurements of DNA damage and is therefore considered as highly reliable (Dhawan et al., 2009; Olive et al., 2012). It is well known that high temperature stress is associated with ROS production and oxidative stress (Hetz and Bradley, 2005; Korsloot et al., 2004; Pörtner and Knust, 2007; Speakman, 2005). DNA damage caused by ROS is mainly due to oxidation of nucleotides. It occurs most readily at guanine residues due to the high oxidation potential of this base relative to cytosine, thymine, and adenine (Cadet and Wagner, 2013). However, the DNA breaks will be transiently present when cells repair lesions via base excision or nucleotide excision, and so a high level of breaks in the comet assay may indicate either high damage or efficient DNA repair system (Collins et al., 1997). The fact that even though measured DNA damage parameters (TL, OTM, %TDNA) in the cells were high, all



insects from this treatment survived show that this species is equipped with very efficient DNA repair system. Slocinska et al. (2013) showed that this species possesses effective mechanisms preventing ROS formation in cockroach muscle and fat body by regulation of free radicals' synthesis. This energy dissipating system might be implicated in cellular protection against metabolic stress in insect tissues. The correct action of these mechanisms has a significant influence on the basic functions of a cell and an organism (Mladenov and Iliakis, 2011). For animals that are under the constant pressure of toxic factors (not only genotoxicological ones), damage repair as well as the synthesis of new molecules to replace damaged ones is extremely important (Augustyniak et al., 2008; Calow and Sibly, 1990; Jha, 2008). We therefore suggest that the death at low freezing temperatures does not occur due to the DNA damage caused by temperature stress but due to other factors i.e. physical ice formation inside the body. The results of the present study broaden the knowledge about the effect of thermal stress on DNA damage in insects. We have showed that SCGE can be an efficient method to analyze the genotoxic effect of different stressors, in our case temperature.

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## 6. Conflict of interest statement

The authors declare that there are no conflicts of interest, financial or otherwise. The funder (National Science Center, Poland) had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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676  
677 Figure captions:

678 **Fig. 1. Scheme of the experimental designs for the survival experiments (A) and comet assay**  
679 **(B)**

680  
681 **Fig. 2. Exemplary supercooling curves for two individual adult males of *G. coquereliana*.** The  
682 temperature ramp was set at a -0,5 °C/min rate to reach target temperature of -30 °C.

683  
684 **Fig. 3. Survival curves of adult males of *G. coquereliana* after COLD, FREEZE and HEAT**  
685 **treatment over 10 days period.** For survival analyses, Mantel-Cox test with mortality at 10  
686 days was used. Each group consisted of 10 individuals.

**Fig. 4. Representative images of effects of temperature stress on DNA damage in haemocytes isolated from adult males of *G. coquereliana* after A) COLD, B) FREEZE and C) HEAT treatments.** Picture D) represents positive control where cells were treated with H<sub>2</sub>O<sub>2</sub> at 100 µM concentration and E) insects from negative control (see materials and methods for details). Scale represents 20 µm. To visualize the DNA the preparations were stained with CYGREEN® Dye.

**Fig. 5. Effects of temperature stress on DNA damage in haemocytes of adult males of *G. coquereliana*.** A) Length of the comet tail (TL), B) Percentage of DNA in the comet tail (%DNAT), C) Olive tail moment (OTM) and D) Percentage of cells with visible comets (%COM) of all experimental animal groups. Mixed effects generalized model (GLMM) was applied for each tested parameter and Holm-adjusted Tukey's pairwise comparison test using R. All data are expressed as mean ±SEM. Different letters on the bars indicate significant differences between means.