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RESEARCH ARTICLE

Thermal stress causes DNA damage and mortality in a tropical insect

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ABSTRACT

Cold tolerance is considered an important factor determining the geographic distribution of insects. We have previously shown that despite its tropical origin, the cockroach *Gromphadorinha coquereliana* is capable of surviving exposures to cold. However, the freezing tolerance of this species had not yet been examined. Low temperature is known to alter membrane 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 (SCGE) assays to determine whether heat, cold and freezing altered hemocyte DNA integrity. The SCP of this species was high and around −4.76°C, which is within the typical range of freezing-tolerant species. Most cockroaches survived to 1 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 levels of DNA damage in hemocytes. 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 present study shows that *G. coquereliana* can be considered as moderately freeze-tolerant, and that extreme low temperature stress can affect DNA integrity, suggesting that this cockroach may possess an efficient DNA repair system.

KEY WORDS: *Gromphadorhina coquereliana*, Cockroach, Freezing, Heat stress, Comet assay, Supercooling point

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 a narrower thermal range than temperate ones (Kellermann et al., 2009). Cold tolerance is considered to be an important factor determining the 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, 2010; Wharton, 2007). To resist when temperature falls below the freezing point, insects usually show two main strategies: freezing tolerance and 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, 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 for freezing-intolerant insects; however, many species die at temperatures well above SCP owing 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 and Angilletta, 2009; Ditrich et al., 2018; Sunday et al., 2012). It results that temperate populations are usually better able to 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 (1993, 1996) described tropical insects as 'opportunistic survivors' regarding cold stress, and with climate change, knowledge about the thermal tolerance of tropical insects is valuable owing to the resulting potential expansions of invasive species (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

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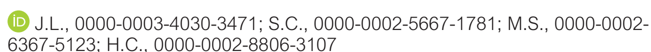
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List of abbreviations

ACB	anticoagulant buffer
OTM	olive tail moment
ROS	reactive oxygen species
SCGE	single cell gel electrophoresis
SCP	supercooling point
TBW	total body water
TL	tail length
%DNAT	percentage of total DNA in the tail
%COM	percentage of cells with visible comets

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 *Cryptocercus punctulatus* and *Celatoblatta quinquemaculata*, which 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 of complex physiological alterations such as loss of ion and water homeostasis, which participate in the disruption of neuromuscular functions, leading to chill coma and, ultimately, death (Overgaard and MacMillan, 2017). Koštál et al. (2006) previously showed that chilling injury resulted in the disturbance of ion homeostasis in the coxal muscle of the tropical cockroach *Nauphoeta cinerea*. We have previously shown that another tropical cockroach from Madagascar, *Gromphadorinha coquereliana*, was surprisingly capable of surviving short-term as well as repeated exposures to low temperatures (Chowański et al., 2017, 2015). In the wild, this species is not supposed to be exposed to cold often, as temperatures only occasionally drop to 4°C for a few hours in Madagascar (Chowański et al., 2015). In spite of this, we found that chilling triggered physiological responses, such as induction of heat shock proteins and aquaporins, or metabolic responses, such as an increase in quantity of total protein in the fat body, higher level of polyols and glucose in hemolymph and changes in mitochondrial respiration activity (Chowański et al., 2017, 2015). The rather high chilling tolerance of *G. coquereliana* is surprising considering the tropical origin of this species (Chowański 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) and xenobiotics (Pietri et al., 2018). Many species exhibit discontinuous respiration, which is supposed to reduce water loss, improving survival of food and water restriction (Schimpf et al., 2012, 2009). Urates play a central role in cockroach physiology. Typically, the fat body contains urocyte cells that contain stored urates (Cochran, 1985; Park et al., 2013). Many studies have confirmed that stored urates serve as an ion sink, allowing for sequestration/release of hemolymph ions as a mechanism for maintenance of ion homeostasis (e.g. Hyatt and Marshall, 1985a,b). This adaptation may be of particular relevance for cold tolerance, as maintenance of ion homeostasis across membranes is a key element of chilling tolerance (reviewed by Overgaard and MacMillan (2017).

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

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

As mentioned above, urate metabolism plays a central role in cockroach physiology (Park et al., 2013). Stored urates (in urocytes) are a particular adaption of cockroaches that contributes to ionic balance and osmoregulation by ion exchanges in tissue fluids (Mullins, 2015; Mullins and Cochran, 1974). Because maintenance of metal ion homeostasis is directly linked to chilling tolerance of insects (Grumiaux et al., 2019; MacMillan et al., 2016; Overgaard and MacMillan, 2017), it is conceivable that this specialization may contribute to the unexpectedly high chilling tolerance of *G. coquereliana* (Chowański et al., 2017, 2015). However, the freezing resistance of this species had not yet been examined. In the present study, we first subjected adult male *G. coquereliana* to freezing temperatures to determine their SCP, and evaluated whether the cockroaches were capable of surviving partial and complete freezing. Next, we conducted single cell gel electrophoresis (SCGE) assays to determine whether heat, cold and freezing stress altered the DNA integrity of hemocytes.

MATERIALS AND METHODS

Insect rearing

Cockroaches [*Gromphadorhina coquereliana* (Saussure 1863)] were reared under laboratory conditions in a continuous colony at 28°C and approximately 65% relative humidity under a 12 h:12 h light:dark cycle in the 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±0.39 cm in size and a mass of 5.5±0.48 g (means±s.d.) were used for experiments.

Determination of supercooling point

To determine SCP, 24 insects were placed individually in 50 ml Falcon tubes, which were submerged in a 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 rate of 0.5°C min^{-1} to reach a target temperature of −30°C. To monitor the temperature of the insects, a

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K-type thermocouple was placed in the middle of the dorsal side of the cockroach, touching the cuticle, secured with Parafilm® and connected to a Testo 175T3 temperature data logger (Testo SE & Co., Germany). The temperature of the insects was recorded every 10 s. The SCP was defined as the temperature at the onset of the freezing exotherm produced by the latent heat.

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 an ongoing decrease in temperature for only 5 min after reaching the SCP, which resulted in incomplete freezing, with a 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 the 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 a 1 liter glass bottle submerged in a 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. For each treatment, three insects were placed in the bottle at the same time. The bottle was secured with a sponge plug in order to prevent the insects from escaping and allowing for the circulation of air. The temperature inside the 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 1 h after each treatment.

Survival

After each thermal treatment, 10 individuals were placed in a breeding room in plastic boxes (15×30×20 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 the same period.

Assessment of DNA integrity of hemocytes

Circulating hemocytes were isolated by collecting hemolymph from single treated individuals. To do so, insects were anesthetized by submersion, as previously described (Chowański et al., 2017). Because hemolymph coagulates rapidly, after anesthesia, insects were injected with 300 µl of anticoagulant buffer (ACB; 69 mmol l^{-1} KCl, 27 mmol l^{-1} NaCl, 2 mmol l^{-1} $NaHCO_3$, 30 mmol l^{-1} sodium citrate, 26 mmol l^{-1} citric acid and 10 mmol l^{-1} EDTA, pH 7.0, Sigma-Aldrich, St Louis, MO, USA) (Chowański et al., 2017, 2015). The ACB was injected under the last pair of legs using a Hamilton syringe (Hamilton Co., Reno, NV, 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, 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 1000 g for 10 min at 4°C. The supernatant was discarded and the hemocytes were resuspended in 100 µl of ACB. Cells were diluted 100× with ACB to obtain ∼2.5×10^5 cells ml^{-1} in all samples. To assess DNA integrity of isolated hemocytes, a Comet SCGE assay kit (ENZO Life Sciences, Inc., New York, NY, USA) was used according to the manufacturer's instructions. Briefly, the cells were combined with molten LMAgarose in a 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 mmol l^{-1} NaOH, 1 mmol l^{-1} EDTA, pH >13) and then washed twice in 1× TBE buffer (80 mmol l^{-1} Tris Base, 89 mmol l^{-1} boric acid, 3.2 mmol l^{-1} EDTA) for 2 min. Slides were then placed flat onto a gel tray and aligned equidistant from the electrodes. The voltage was set to 1 V cm^{-1} (measured electrode to electrode) and applied for 10 min. After electrophoresis, samples were dipped in 70% ethanol for 5 min and dried in an incubator set to 37°C. Comets were stained with 10× CYGREEN® Dye for 30 min and visualized using an Olympus BX41 epifluorescence microscope (Olympus, Tokyo, Japan, FITC filter, excitation/emission 489/515 nm) equipped with a 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_2O_2 was prepared for every electrophoretic run. The cells, which were isolated from randomly selected control animals, were treated with H_2O_2 (100 µmol l^{-1}, Sigma-Aldrich) for 10 min at 4°C, after which they were tested for DNA damage under the same conditions as described above. For each thermal treatment, five or six different treated animals were used; each produced one slide of hemocytes to analyze. In addition, five untreated (unstressed) insects were also used to assess DNA integrity

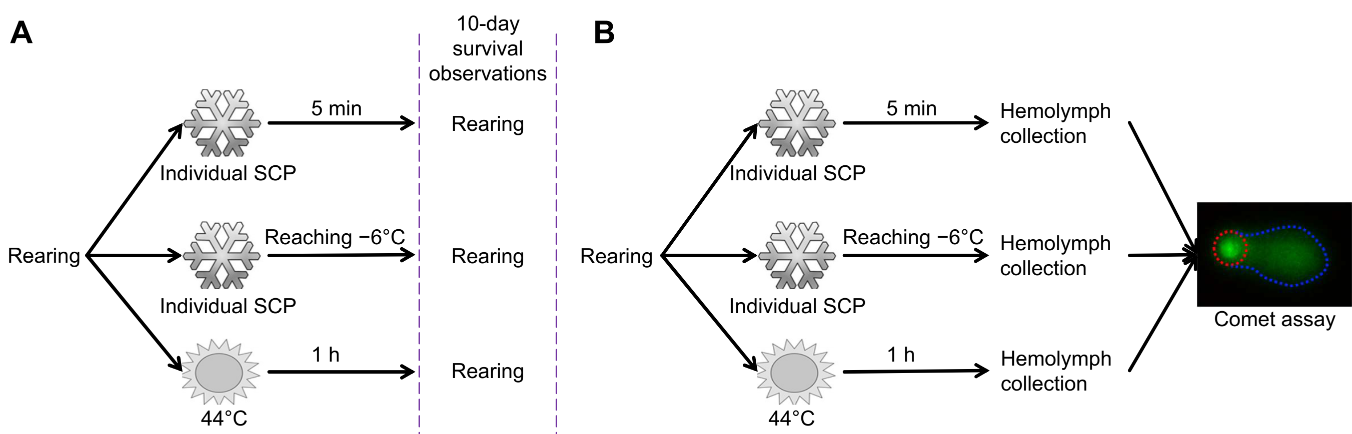

Fig. 1. Scheme of the experimental designs. (A) Survival experiments; (B) comet assay. SCP, supercooling point.

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of their hemocytes, 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).

Image analyses

All images were analyzed with CASP Lab software (Końca et al., 2003). Tail DNA is the most commonly used parameter to assess DNA integrity, but 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).

Statistical analysis

For survival analyses, the Mantel–Cox test with mortality at 10 days was used. For all tests, *P*-values lower than 0.05 were considered statistically significant. The data are presented as means±s.e.m. 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, because 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 a random variable in the model. Therefore, a 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).

RESULTS

Determination of the SCP and survival of stress treatments

SCPs of adult males varied from −7.6 to −1.9°C, with an average of −4.76±1.60°C (*n*=24), and the mean time to complete freezing was 18.0±11.38 min. The SCPs of individuals after cold and freeze treatments were −4.78±1.00°C (min. −6.00°C, max. −2.80°C) and −4.26±1.56°C (min. −6.30°C, max. −2.30°C), respectively. Fig. 2 shows typical cooling curves of *G. coquereliana* with exotherms. Eighty percent 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 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 that of the control group (χ^2=10.59, *P*=0.001). Thirty percent of insects from the freeze group survived 24 h after complete freezing. However, they showed clear signs of injuries, such as inability to walk, feeble movements of appendages, 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 that of the control group (χ^2=17.20; *P*<0.001). During the heat treatment, all

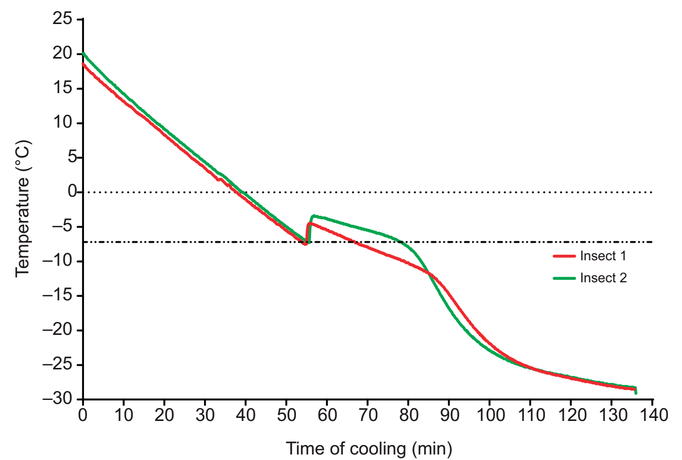

Fig. 2. Example supercooling curves for two individual adult male *Gromphadorinha coquereliana*. The temperature ramp was set at a rate of −0.5°C min^{-1} to reach a target temperature of −30°C. Dotted line, 0°C; dot–dashed line, SCP.

insects showed significant signs of stress: uncoordinated movement, as well as increased abdominal ventilation frequency and defecation. In spite of this, all cockroaches survived the heat treatment for 10 days, showing no statistical differences compared with the control treatment (*P*>0.05).

DNA damage

Fig. 4 shows the effects of treatments on the isolated hemoctyes and Fig. 5 the results of all of the DNA damage metrics according to the three thermal treatments, the untreated control and the positive H_2O_2-treated control. The mean values differed significantly according to treatments in all tested parameters (TL: χ^2=6496.2, d.f.=4, *P*<0.001; %DNAT: χ^2=6968.5, d.f.=4, *P*<0.001; OTM: χ^2=5859.6, d.f.=4, *P*<0.001; %COM: χ^2=6441.4, d.f.=4, *P*<0.001). These differences were mainly driven by H_2O_2-treated samples that, as expected, showed substantial DNA damage. Pairwise multiple comparisons were used to discriminate significance of the different treatments, as indicated by different letters in Fig. 5. For all tested parameters, comparisons showed that DNA damage was much greater in H_2O_2 samples than in the other treatments (Tukey tests, *P*<0.001). Values of freeze or heat treatments were lower than in the

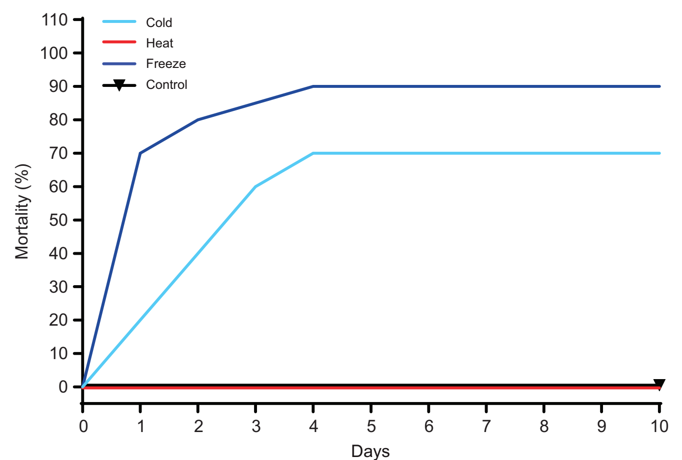

Fig. 3. Survival curves of adult male *G. coquereliana* after cold, freeze and heat treatment over 10 days. For survival analyses, Mantel–Cox test with mortality at 10 days was used. Each group consisted of 10 individuals.

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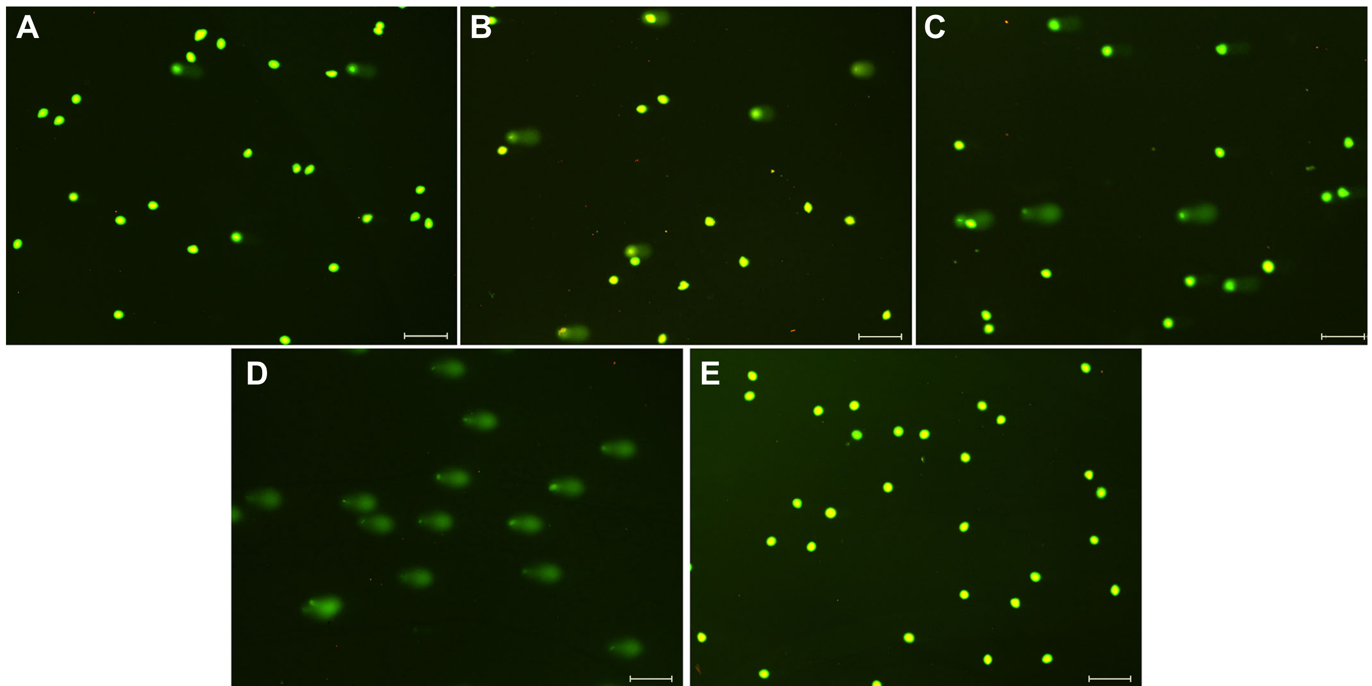

Fig. 4. Representative images of effects of temperature stress on DNA damage in hemocytes isolated from adult male *G. coquereliana*. Data are shown after (A) cold, (B) freeze and (C) heat treatments. (D) Positive control, where cells were treated with H_2O_2 at 100 µmol l^{-1} concentration. (E) Insects from negative control (see Materials and Methods for details). Scale bars: 20 µm. To visualize the DNA, the preparations were stained with CYGREEN® Dye.

H_2O_2 treatment, but clearly greater than that of the 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 the control treatment (Tukey tests, $P<0.001$).

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. We also show for the first time that cold stress does not substantially damage the DNA whereas extreme low temperature stress can affect DNA integrity in hemocytes 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°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 below zero (−4.7±1.6°C). This is within the typical range of freezing-tolerant cockroach species, such as the alpine cockroach, *Celatoblatta quinquemaculata*, or the Japanese cockroach, *Periplaneta japonica*, which 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 the contrary, freeze-avoiding insects generally exhibit deep supercooling ability, with SCP values often in the range of −15 to −25°C or lower (Danks, 2004; Vernon and Vannier, 2002). Although the SCP profile of *G. coquereliana* looks exactly as those reported in the freezing-tolerant *C. quinquemaculata* (Worland et al., 1997, 2004), it would be surprising if *G. coquereliana* had developed (or inherited) physiological adaptations to survive freezing. The Blattodea is a 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 temperate or tropical zones; however, according to Sinclair et al. (2003a), they are mostly freeze-tolerant insects (Sinclair et al., 2003a). Hence, cold adaption may be an ancestral heritage within this order. Alternatively, the freeze tolerance of *G. coquereliana* 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 died when body temperature was further reduced after onset of freezing. So it can be classified as moderately freeze 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 ca. −2.5°C and survives frozen to ca. −8°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 of 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

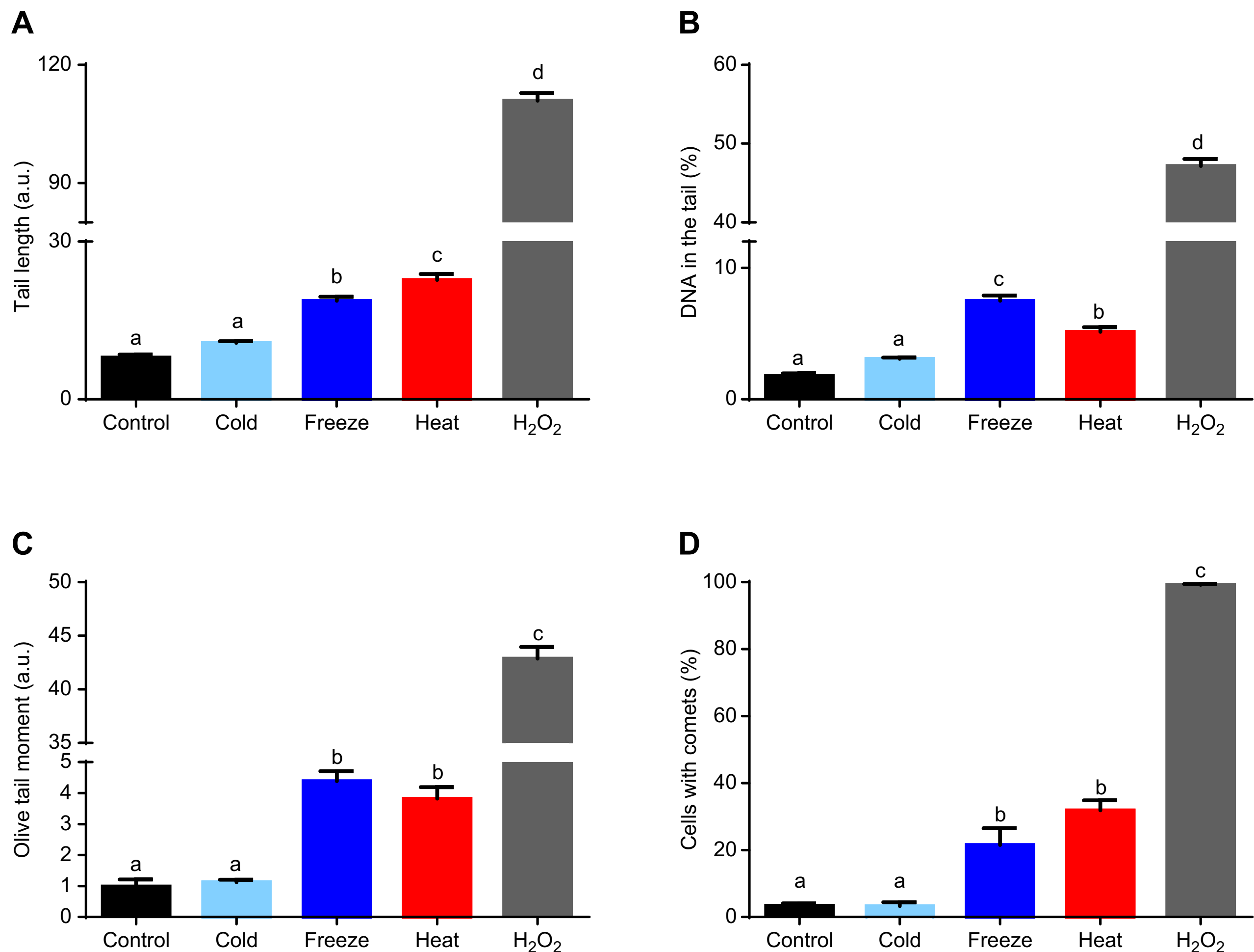

Fig. 5. Effects of temperature stress on DNA damage in hemocytes of adult male *G. coquereliana*. (A) Length of the comet tail (TL), (B) percentage of total DNA in the comet tail (%DNAT), (C) olive tail moment (OTM) and (D) percentage of cells with visible comets (%COM) of all experimental animal groups.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

(Muldrew et al., 2004; Overgaard and MacMillan, 2017; Overgaard et al., 2005). In *M. domestica*, cold stress was reported to lead to an increase in chromosome aberrations and micronucleus frequency occurrence (Mishra and Tewari, 2014). Until now, whether cold and freezing stress can damage DNA had not yet been examined in any insect models.

In the present study, when cockroaches were heat-stressed, the observed DNA damage was similar to that from the 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 greater in both treatments compared with the control. OTM incorporates quantitative and qualitative measurements of DNA damage and is therefore considered to be 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 owing 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 an efficient DNA repair system (Collins et al., 1997). The fact that all insects from this treatment survived, even though measured DNA damage parameters (TL, OTM, %TDNA) in the cells were high, shows that this species is equipped with a very efficient DNA repair system. Slocinska et al. (2013) showed that this species possesses effective mechanisms preventing ROS formation in the muscle and fat body by regulation of the synthesis of free radicals. 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 cells and organisms (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 are 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 as a result of the DNA damage caused by temperature stress but rather because of 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 shown that SCGE can be an efficient method to analyze the genotoxic effect of different stressors, in our case, temperature.

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Competing interests
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.

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

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