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RESEARCH ARTICLE

Mating under climate change: Impact of simulated heatwaves on the reproduction of model pollinators

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Abstract

1. Climate change is related to an increase in frequency and intensity of extreme events such as heatwaves. It is well established that such events may worsen the current world-wide biodiversity decline. In many organisms, heat stress is associated with direct physiological perturbations and could lead to a decrease of fitness. In contrast to endotherms, heat stress resistance has been poorly investigated in heterotherms; especially in insects, in which the internal physiological mechanisms available to regulate body temperature are almost negligible making them sensitive to extreme temperature variations.
2. Wild bees are crucial pollinators for wild plants and crops. Among them, bumblebees are experiencing a strong decline across the world. Therefore, the ongoing global decline of these insect pollinators partly due to climate change could cause major economic issues.
3. Here we assess how simulated heatwaves impact fertility and attractiveness (key parameters of sustainability) of bumblebee males. We used three model species: *Bombus terrestris*, a widespread and warm-adapted species, *B. magnus* and *B. jonellus*, two declining and cold-adapted species.
4. We highlight that heat shock (40°C) negatively affects sperm viability and sperm DNA integrity only in the two cold-adapted species. Heat shock can also impact the structure of cephalic labial glands and the production of pheromones only in the declining species.
5. The specific disruption in key reproductive traits we identify following simulated heatwave conditions could provide one important mechanistic explanation for why some pollinators are in decline through climate change.

KEYWORDS
attractiveness, climate change, fertility, heterotherms, pollinators, simulated heatwaves

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1 | INTRODUCTION

Global biodiversity is undergoing a strong decline because of global changes induced by anthropogenic pressures (Dirzo et al., 2014). Among these disturbances, climate change will impact ecosystems by modifying the geographic range of species (Fitter & Fitter, 2002; Hance et al., 2007; Parmesan et al., 2000) and by fostering local extinctions or new biological invasions (Hoffmann et al., 2013). These impacts have mainly been assessed by considering slow and gradual increase of isotherms and global mean temperatures. However, the ongoing climate change also triggers an increase of extreme events such as heatwaves (Easterling et al., 2000; Lemos & Rood, 2010; Meehl & Tebaldi, 2004; Ragone et al., 2018; Russo et al., 2015). Such events may worsen the current biodiversity decline because (a) their swiftness may leave no chance to organisms for any adaptation (Huntley et al., 2010) and (b) they induce short, stochastic and unusually extreme thermal conditions which negatively impact biological functions (Bridle & Vines, 2007). Notwithstanding, the consequences of heatwaves or heat shock on environments and biodiversity have been poorly investigated to date (Palmer et al., 2017; Ummenhofer & Meehl, 2017), and we have little knowledge of the proximate mechanisms by which climate change could induce extinctions in such a diverse group like insects (Cahill et al., 2013).

Insects (i.e. mainly ectotherm taxa) are particularly sensitive to extreme ambient temperature variations (Deutsch et al., 2008; Kingsolver et al., 2013). Although thermal sensitivity is species-specific (Chen et al., 2011; Deutsch et al., 2008; Huang et al., 2006; Rohmer et al., 2004), overall, such variations lead to direct physiological perturbations (e.g. ontogenic development, water balance and immunity decrease) and, possibly, to the death of individuals (Kingsolver et al., 2013; Neven, 2000; Parmesan, 2006). The ongoing global decline of insect populations partly due to climate change could cause major economic issues since among them pollinators play a key eco-systemic role for human activities in biotic pollination of flowering plants (Goulson et al., 2005; Ollerton et al., 2011; Potts et al., 2016).

Until now, consequences of heat shock on biological reproduction have been poorly investigated in insects. It has been shown that high temperatures can have effects on courtship behaviours (i.e. wing vibrations and odour profiles involved in pre-mating recognition) of solitary bees *Osmia* (Conrad et al., 2017) and could also affect communication in insects and thus the associated behaviours (Boullis et al., 2016). Moreover, previous studies in flies *Drosophila*, beetles *Tribolium castaneum*, parasitic wasps *Anisopteromalus calandrae* and domesticated bees *Apis mellifera* showed a decrease in male reproductive success associated with environmental changes including heat shocks (David et al., 2005; Hurley et al., 2018; Nguyen et al., 2013; Rohmer et al., 2004; Sales et al., 2018; Stürup et al., 2013). Indeed, spermatozoa are very sensitive to heat exposure (Birkhead et al., 2009) and even small temperature variations can impact fertility (Burfening et al., 1970). This phenomenon can be especially deleterious for some insect groups such as Hymenoptera because (a) in social Hymenoptera, monandry is common and females store the sperm in a spermatheca for several weeks/months and even for several years in some groups (e.g. ants) until laying (Chown et al., 2010; Fox & Reed, 2011) and (b) males of some species have a single stock of spermatozoa produced at their emergence (Duvoisin et al., 1999). Alteration of this stock could therefore have dramatic consequences on reproductive function of males. Spermatozoa lodged in spermatheca of female Hymenoptera can remain alive several months with a low mortality rate and a low level of alterations (Greeff & Schmid-Hempel, 2008) although this stock remains sensitive to heatwave exposure (Sales et al., 2018). Therefore, temperatures may have a major impact on sexual selection in bee species such as in the genus *Bombus*.

Bumblebees (*Bombus*) constitute a group of crucial pollinators in temperate and polar regions of North America and Eurasia for wild plants and crops (Garibaldi et al., 2013; Potts et al., 2010). Much attention is addressed for management and conservation of domesticated bees (i.e. *Apis mellifera*) for crops. However, a considerable part of the pollination service for wild plants is supported by non-managed bees such as bumblebees which received much less attention. Because of intrinsic differences (e.g. physiological, behavioural, etc.), responses to environmental stressors of managed honeybees should not be extrapolated for wild bees (Woods et al., 2020). Many bumblebee species are showing negative population trends partly explained by climate change (Kerr et al., 2015; Nieto et al., 2014; Rasmont et al., 2015; Soroye et al., 2020). While they are robust and hairy endo-heterothermic bees, they seem highly sensitive to extreme temperatures (Heinrich, 2005; Martinet et al., 2015; Oyen et al., 2016), as highlighted by behavioural adaptations exhibited by workers to cool colonies down (e.g. wing fanning; Vogt, 1986). Bumblebees, as most of Hymenoptera, are an ideal model to study male fertility disruptions because of their haplo-diploid sex determination: diploid female emerges from a fertilized egg, while haploid male emerges from a non-fertilized egg (Baer, 2003). Their ability to produce daughters, and consequently their F1 population dynamics, depends on the availability of stored sperm (clonal sperm provided by a unique haploid male) in the spermatheca of the queen after mating (Baer, 2003). Before reproduction, most bumblebee males perform a nuptial behaviour (e.g. patrolling) to attract virgin queens. During this courtship, they emit pheromones known as Cephalic Labial Gland Secretions (CLGS; Ayasse & Jarau, 2014; Calam, 1969; Valterová et al., 2019). These secretions are species-specific, synthesized de novo (Žáček et al., 2013) and intended to attract females before mating (Bergström et al., 1981). It has been suggested that a slight modification in pheromone mixtures may impede conspecific recognition of individuals (Bergström et al., 1981; Boullis et al., 2016; Dobzhansky & Gould, 1982; Lecocq et al., 2015).

In this study, we investigated the impact of heat shock on reproduction of key pollinators in our ecosystems (i.e. bumblebees). We assessed on two cold-adapted species (*Bombus jonellus* and *Bombus magnus*) and one Euro-Mediterranean species (*Bombus terrestris*) (a) the quality of sperm (viability and DNA degradation); and (b) the histology and chemical composition of their attractive cephalic

labial glands (i.e. pheromonal glands). We expect that structure of the cephalic labial gland to be impacted by heat shock involving a dysfunction in the secretion of pheromones and ultimately a shift in the attractiveness of males. Heat exposure should also induce a reduction of bumblebee's fertility. Based on these hypotheses, if attractiveness is not impacted by heat stress, females could mate with males having low sperm quality and population fitness would be reduced. We also expected that male reproductive traits will be more impacted in more cold-adapted species than in the resistant one, explaining specific population trends of bumblebees under climate change.

2 | MATERIALS AND METHODS

2.1 | Studied species

Three European species were selected for this study: *Bombus terrestris* (the buff-tailed bumblebee), *B. jonellus* (the heath bumblebee) and *B. magnus* (the northern white-tailed bumblebee). The first species is a Euro-Mediterranean species (heat-tolerant), spreading northward in Europe (Rasmont et al., 2015) and invasive in many parts of the world (Inari et al., 2005; Torretta et al., 2006). The two other species are cold-climate adapted declining in many parts of their distribution (Nieto et al., 2014) and live in some areas of temperate regions (e.g. in the heath landscapes of Belgium). These two species have been selected based on their potential high sensitivity to heat shock (Martinet et al., 2015; Rasmont et al., 2015). For all species, we have collected queens in the field and brought them back to the laboratory in optimal breeding conditions (see below 'colonies breeding'). Females were collected during their optimal life stage for breeding (just after their hibernation in early spring). All individuals were collected into the wild from two Belgian localities in 2017 and 2018 (Kalmthout, 51°22′N, 04°28′E 21 m alt., and Maasmechelen, 50°57′N, 05°41′E 39 m alt.). We reared the different colonies in plastic boxes (8 cm × 16 cm × 16 cm) provided by Biobest NV at their optimal temperature of 24–26°C with 50%–60% of relative humidity (e.g. Vanderplanck et al., 2019). Colonies were maintained in constant darkness and were fed with BIOGLUC (Biobest NV) as sugar resources and pollen of *Salix* sp. as lipids/proteins resources (assumed as a highly suitable diet; Vanderplanck et al., 2019). Pollen loads were purchased from the '*Ruchers de Lorraine*' company. New pollen candy (pollen mixed with BIOGLUC) was provided every 2 days, while the previous one was removed before decaying. From queen-right colonies (colonies in development with a queen), we have produced males under laboratory conditions to have individuals of equal age (7–10 days) for the different analyses and experimentations to avoid potential bias of emerging age in sperm quality and composition of pheromones (Coppée et al., 2011). To ensure that all the males were exposed to the exact same conditions before our experiments, we maintained all males at 26°C and 50%–60% relative humidity directly after emergence until their maturity. All males were 7–10 days old at the time of the experiment; this period corresponds to the maximum production of pheromones of mature males able to mate with females (Ayasse & Jarau, 2014; Baer, 2003; Coppée et al., 2011). Males of these three bumblebee species display the same nuptial behaviour (i.e. patrolling behaviour; Ayasse & Jarau, 2014).

2.2 | Simulated heatwave conditions

The protocol used to simulate heatwave stress is based on methodology of Martinet et al. (2015). To standardize experimental conditions, all specimens (heat stressed and non-stressed males) were maintained overnight at the standby temperature of 8°C in the dark with sugar syrup (Biogluc; Biobest) before performing the experiments. One hour before experimentation, all males were warmed up at 24°C. Then, they were placed in controlled and constant conditions in an incubator (Herp Nursery II) at 40°C with a humidity rate of 50%–60%. The temperature of 40°C was chosen to simulate a heatwave temperature which has been recorded in natural environments across more than 100 countries (Mherrera, 2016). In our challenge test, we calculated 'Time before Heat Stupor' (THS) as an estimator of individual heat resistance. THS corresponds to the time between the introduction of specimen into the incubator until heat stupor, event during which insect falls on its back, failing to right itself and loses its normal reflexes (critical motor function; Bodenheimer & Klein, 1930; Huang et al., 2006; Uvarov, 1931). After entering in heat stupor (heat shock), individuals were removed from the experimental device for recovery (4–5 min). Heat stressed males (heat stressed group) and non-stressed males maintained at 26°C (control group) were tested the same day. For heat resistance tests, we used males of *B. jonellus* ($n = 30$), *B. magnus* ($n = 30$) and *B. terrestris* ($n = 30$). For all the experiments described below, specimens collected have been separated into two groups: (a) heat stressed group (exposed at 40°C until heat stupor state) and (b) control group (maintained at optimal conditions 26°C with 50%–60% of relative humidity). We used males from different colonies to avoid the 'colony relating effect'. The temperature and relative humidity were controlled and kept constant. Colonies were fed with pollen of *Salix* sp. Sugar resources were supplied by sugar syrup (Biogluc; Biobest). Heat stressed groups and control groups were exposed to the same conditions to avoid bias in interpretation linked to different temperature conditions between control and heat stressed groups or acclimation.

2.3 | Heat shock impacts on sperm viability and sperm DNA integrity

2.3.1 | Sperm extraction

Sperm was extracted from bumblebee males as follows. Individuals were held by the thorax and rolled between thumb and forefinger for a few seconds to relax them. Sternite 1 was pressed gently with the thumb on his full-length a couple of times to stimulate males.

Thereafter, the sternite 4 and 5 were pressed harder with a movement from sternite 4 to sternite 5 until the outgoing of genitalia. At this moment, the last sternite was pressed to expulse entirely the genitalia including the endophallus filled with sperm. With a pair of tweezers, the base of the genitalia was pinched and gently pulled out of the abdominal cavity together with testis and accessory glands. The genitalia were directly transferred in a Petri dish and covered with 70 µl of sodium phosphate buffer (PBS solution, pH 7.4). Genitalia were then crushed in this solution using the scalpel' blade to extract sperm. Finally, sperm was transferred into microtubes before mixing with agarose for sperm DNA degradation test at room temperature.

2.3.2 | Sperm viability assessment

Sperm viability was assessed using the FluoVit kit (Microptic) following the manufacturer's instructions. Briefly, 10 µl of semen samples were mixed with 1 µl of BLUE component (Hoechst and trihydrochloride trihydrate) and incubated for 5 min following kit's instructions. After addition of 1 µl of RED component (Propidium Iodide), 10 µl of stained samples were mounted with Vectashield (Vector Laboratories) and observed by using a Zeiss Axioscope A1 microscope. Propidium iodide fluorescence was excited using a 562 ± 20 nm excitation filter, and emitted fluorescence collected with a 624 ± 20 nm filter. Blue fluorescence was excited using a DAPI filter using a 330–380 nm excitation filter, and emitted fluorescence collected with a 400–420 nm filter. Alive spermatozoa were stained in blue, while dead spermatozoa were stained in red. For each group (heat stressed or control group, as explained above), two pseudo replicas were done for each specimen. In total, 200 spermatozoa were counted by replicas (=400 spermatozoa per specimen). The proportion of viable sperm in each specimen was calculated as the average (across the two pseudo replicas) of the total number of live spermatozoa, divided by the total number of counted spermatozoa. The total number of spermatozoa counted was included as a fixed factor in a generalized linear mixed model (Bolker et al., 2009). For this test, we used *B. terrestris* (heat stressed group $n = 37$; control group $n = 45$), *B. magnus* (heat stressed group $n = 30$; control group $n = 28$) and *B. jonellus* (heat stressed group $n = 16$; control group $n = 15$).

2.3.3 | Sperm chromatin dispersion assessment

In addition to viability and motility of sperm, for 30 years, researchers have been investigating impact of sperm DNA fragmentation on the success of fertilization (Borsuk et al., 2018; Evenson et al., 1980; Evenson & Wixon, 2005; Fernández et al., 2003; Gosálvez et al., 2010). Numerous studies indicate that in some human patients, DNA is altered and have single- and double-strand breaks (Lewis et al., 2013; McEvoy et al., 2014; Nicopoullos et al., 2008). Consequences of this fragmentation on reproduction capacities are controversial, but some analyses indicate a positive correlation between this fragmentation and a lower rate of pregnancies (Boe-Hansen et al., 2006; Rex et al., 2017). Despite all the biological mechanisms to protect sperm, DNA fragmentation induced by environmental stresses (e.g. heat stress; Borsuk et al., 2018) may occur. High temperatures activate nucleases present in the spermatic fluid, and therefore increase the rate of chromatin fragmentation (Pruski et al., 2009). Sperm Chromatin Dispersion test (SCD) evaluates DNA fragmentation (i.e. presence of DNA single- or double-strand breaks) of sperm (and indirectly the fertility) by using the chromatin denaturing capacity. During this test, spermatozoa are treated with an acid solution to denature DNA that contains breaks and then treated with a lysis solution which removes membranes and proteins (including nuclear proteins). As a result, the remaining (non-fragmented) DNA forms nuclear-matrix-linked loops (Lewis et al., 2013). The size of the halo formed by these loops around nucleus represents the amount of non-fragmented sperm DNA; the larger the halo the lower the fragmentation (Lewis et al., 2013).

In this study, SCD test was adapted from Fernández et al. (2003) and Gutiérrez et al. (2007). Briefly, 30 µl of semen samples (PBS + sperm) was mixed with 70 µl of 1% low melting agarose and 25 µl of the resulting solution were spread onto slides pre-coated with agarose. After solidification for 10 min at 4°C, the slides were immersed in denaturing solution (0.08N HCl) for 7 min, then in lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 5 mM TCEP, 0.5% Triton, pH 7.4) for 25 min and finally in MilliQ water for 5 min. The lysis solution used in the kit allows excluding protamines and other non-nuclear matrix proteins, which has the effect of deploying the remaining (non-fragmented) DNA in the form of nuclear-matrix-linked loops (Lewis et al., 2013; Nicopoullos et al., 2008; Shaman et al., 2007). To visualize released DNA, the use of a DNA marker or a staining is required (DIFF-QUICK staining). The slides were then dehydrated in increasing concentrations of ethanol, dried for 5 min and stained using the Diff-Quik kit (Microptics) following the manufacturer's instructions. The stained slides were mounted using ROTI-Histokit II kit (Carl Roth), dried horizontally overnight at room temperature and observed by using a Zeiss Axioscope A1 microscope. The level of fragmentation of sperm DNA was classified in four categories according to the size of the halo with ImageJ: 'full halo' (=non-fragmented DNA), 'degraded halo' (=fragmented DNA), 'halo-free' (=fragmented DNA) and 'sperm without flagella'. For each group (control and heat stressed groups) four pseudo replicas were done for each specimen. In total, 200 spermatozoa were counted by replicas (=800 spermatozoa per specimen). Proportion of DNA fragmentation in each specimen was calculated as the average (across the four pseudo replicas) of the total number of degraded sperms divided by the total number of counted sperms. The total number of spermatozoa counted was included as a fixed factor in a generalized linear mixed model (Bolker et al., 2009). For this test, we used *B. terrestris* (heat stressed group $n = 16$; control group $n = 15$), *B. magnus* (heat stressed group $n = 15$; control group $n = 15$) and *B. jonellus* (heat stressed group $n = 16$; control group $n = 15$).

2.4 | Hyperthermic shock impacts on attractiveness of males

2.4.1 | Histology of the cephalic labial glands

For histological analyses, samples of freshly dissected heads were fixed in Bouin's liquid for 48 hr. They were then transferred to a decalcifying solution (5% trichloroacetic acid with 5% formaldehyde) for 2 days. Samples were dehydrated in graded ethanol, embedded using a routine method in paraffin wax (Gabe, 1968) and sectioned at a thickness of 5 µm with a Leica RM2145 microtome. Sections were stained with Masson's Trichrome and observed by using a Zeiss Axioscope A1 microscope. We separated degraded acini from non-degraded acini based on the form of lumen of acini. For this test, we used *B. terrestris* (heat stressed group $n = 5$; control group $n = 5$) and *B. jonellus* (heat stressed group $n = 5$; control group $n = 5$) with three pseudo replicas for each specimen.

2.4.2 | Analysis of cephalic labial gland secretions

Cephalic Labial Gland Secretions (CLGS) were extracted in 400 µl of n-heptane, according to the method described by De Meulemeester et al. (2011). The qualitative composition of CLGS was determined by gas chromatography-mass spectrometry using a Finnigan GCQ quadrupole system (GC/MS) with a non-polar DB-5 ms capillary column (5% phenyl (methyl) polysiloxane stationary phase; column length 30 m; inner diameter 0.25 mm; film thickness 0.25 µm). The identification of all characterized compounds was confirmed with analytical standards. Relative quantification was performed for all CLGS samples with a gas chromatograph Shimadzu GC-2010 system (GC-FID) equipped with a non-polar SLB-5 ms capillary column (5% phenyl (methyl) polysiloxane stationary phase; column length 30 m; inner diameter 0.25 mm; film thickness 0.25 µm) and a flame ionization detector. For this test, we used *B. terrestris* (heat stressed group $n = 15$; control group $n = 15$) and *B. jonellus* (heat stressed group $n = 15$; control group $n = 10$).

2.5 | Statistical analyses

Data were analysed using R 3.5.1 (R Development Core Team, 2018). Graphs were produced using 'GGPLOT{GGPLOT2}' (Wickham et al., 2016) package within R. Descriptive statistics ($M \pm SE$) were calculated by 'DESCRIBEBY{PSYCH}' (Revelle, 2016). A Kruskal–Wallis test was performed to show difference between heat stress resistance among species followed by a post hoc Kruskal–Wallis multiple to identify which species are different from the others.

For sperm (viability and sperm DNA fragmentation), exploratory analysis included distribution plotting and conservative nonparametric testing on ranks prior to fitting generalized linear models (GLMs) with 'GLM{STATS}' (Bates et al., 2014). For replicates, either group averages were calculated, or generalized linear mixed models (GLMMs) were fitted, using 'GLMER{LME4}' (Bates et al., 2014). The most appropriate error distribution for each GLM(M) was selected by examining diagnostic residual plots using 'PLOT{GRAPHICS}' and 'MCP.FNC {LMER CONVENIENCE FUNCTIONS}' (Tremblay & Ransijn, 2015). Proportion response variables, which included sperm viability and sperm DNA fragmentation were fitted using a binomial distribution and a logit link function. After each maximal model was fitted, the statistical significance of the experimental treatment variables was assessed using Akaike's information criterion (AIC) comparisons, and log likelihood ratio tests (LLRT) with, and without, the term of interest (Johnson & Omland, 2004). The most efficient models had significantly lower AICs (Ver Hoef & Boveng, 2007). LLRTs were χ^2 tests when the response variable was a count or proportion, and F tests when continuous. Post hoc pairwise Tukey comparison tests between treated groups and controls were applied using 'LSMEANS {LSMEANS}' (Lenth & Length, 2018). As a measure of how much variation in the response variable was explained by the model, pseudo R^2 (explained deviance) was calculated for GLMs. For GLMMs, 'R. SQUAREDGLMM' (Tremblay & Ransijn, 2015) reported the marginal R^2 explained by the fixed factors, and conditional R^2 for the fixed factors.

Statistical comparative analyses of CLGS were performed using R 3.5.1 (R Development Core Team, 2018) to detect CLGS differentiation. Data were transformed [log (x + 1)] to reduce great difference of abundance between highly and lowly concentrated compounds. A principal component analysis (PCA, R-package MASS; Ripley et al., 2013) was used based on correlation distance matrices, and a clustering method computed with the unweighted pair-group method with average linkage (UPGMA) based on Canberra distance matrices (RA of each compound; R package APE; Paradis et al., 2004). The uncertainty in hierarchical cluster analysis was assessed using p-values calculated by multiscale bootstrap resampling with 100,000 bootstrap replications (significant branch supports >0.85; R package PVCLUST; Suzuki & Shimodaira, 2011). CLGS divergences between taxa were also assessed by performing a multiple response permutation procedure (MRPP, R package VEGAN; Oksanen et al., 2013) based on groups identified by hierarchical cluster analysis. When a significant difference was detected, pairwise multiple comparisons were performed with an adjustment of p-values (Bonferroni correction) to avoid type I errors.

3 | RESULTS AND DISCUSSION

Extreme heat stress impacts organisms on different levels, affecting their physiology, nuptial behaviour, life expectancy or fertility (Hance et al., 2007; Kingsolver et al., 2013; Uvarov, 1931) and in different intensities depending on species traits (Garcia-Robledo et al., 2016). First, we investigated the sensitivity to heat shock of the three studied species by measuring the time needed for individuals exposed to 40°C (a heatwave temperature which has been recorded in natural environments across more than 100 countries; Mherrera, 2016; Robinson, 2001) to reach the heat stupor. In recent years, this temperature of 40°C has been reached and even

exceeded every year in the bumblebee species' native range, including in Belgium where the present study was conducted. We showed that *B. jonellus* and *B. magnus* reached heat stupor after less than 60 min while *B. terrestris* reached heat stupor after more than 400 min (Figure 1). In our test case, heat stress resistance appears to be different among species as the two-declining cold-climate species, *B. jonellus* and *B. magnus*, were significantly more impacted than the invasive Euro-Mediterranean species, *B. terrestris* ($p < 0.01$, Figure 1). Despite surviving exposure to heat shock, the reproductive potential of survivors could still be affected (e.g. a decrease in male fertility and attractiveness; Mironidis & Savopoulou-Soultani, 2010; Roux et al., 2010).

3.1 | Effect of hyperthermic shock on sperm viability and sperm DNA integrity

Bumblebee males exposed to a simulated heatwave condition until heat stupor state (heat stressed group) showed a drastic decrease in sperm viability compared to control group (Figure 2A,B; see Table S1) as observed in *Apis mellifera* after heat exposure (McAfee et al., 2020; Stürup et al., 2013). Although all species were impacted by thermal stress, damages on sperm were significant for sensitive species (i.e. *B. jonellus* and *B. magnus*, $p < 0.01$) but not for the resistant and widespread species *B. terrestris* ($p = 0.63$). In non-stressed males, viable spermatozoa accounted for more than 70% of spermatozoa in *B. terrestris* and *B. jonellus* and for more than 60% of spermatozoa in *B. magnus* (Figure 2A). In heat stressed males, less than 20% of total spermatozoa assessed were still viable in *B. jonellus*

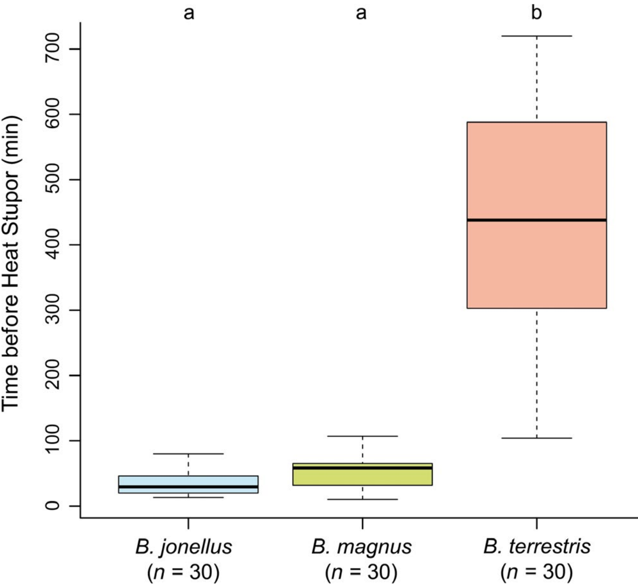

FIGURE 1 Heat stress resistance of the three bumblebee model species. Boxplots representing the time needed to reach heat stupor in the three model species: *Bombus jonellus* ($n = 30$), *B. magnus* ($n = 30$) and *B. terrestris* ($n = 30$). Letters above the boxplots represent groups supported statistically (Kruskal–Wallis test)

and *B. magnus*, while more than 60% of spermatozoa were viable in *B. terrestris*. For the viability test, basal rates of mortality (~30%) in control groups were not significantly different among bumblebee species ($p = 0.28$). Death of spermatozoa could be explained by oxidative stress induced by heat stress. Oxidative stress is defined as the presence of reactive species in excess of the available antioxidant capacity of animal cells. It is known that reactive species can modify several biological cellular macromolecules and can interfere with cell signalling pathways in poultry or in mammals (Akbarian et al., 2016; Altan et al., 2003; Belhadj Slimen et al., 2014), although data on Hymenoptera are missing.

In addition to the decrease in sperm viability, we observed an increase in rate of sperm with fragmented DNA, from 20% for control group to 41% for heat stressed group for *B. jonellus* ($p < 0.01$) and from 18% to 43% for *B. magnus* ($p < 0.01$). Concerning *B. terrestris*, the percentage of sperm with fragmented DNA appeared to increase (from 12% for control group to 17% for heat stressed group), although the difference was not significant ($p = 0.20$; Figure 2C,D). Noteworthy, the basal rate of sperm with degraded DNA (~15%) in individuals from control groups was not significantly different between bumblebee species ($p = 0.36$). All data are available in Supplementary File 2.

In a large panel of species (including insects), several studies show that sperm DNA fragmentation induced by different abiotic or biotic stress could be correlated to a decrease of fertilization success (Evenson & Wixon, 2005; Fernández et al., 2003; Gosálvez et al., 2010; Gutiérrez et al., 2007; Johnston et al., 2017; Lewis et al., 2013; Nicopoullos et al., 2008; Osman et al., 2015; Pollock et al., 2015; Pruski et al., 2009; Sakkas et al., 1999; Sharma et al., 2004; Wright et al., 2014). Therefore, this parameter could be biologically relevant to study the effect of environmental stress on sperm quality. However, complementary studies (e.g. fecundation test) are required to assess if degraded DNA of sperm is deleterious for fertility in bumblebees.

Damages of high temperatures on cells have already been reported as species-specific (e.g. *Mytilus* sp.; Yao & Somero, 2012). In sperm cells of mice, high temperatures activate nucleases present in the spermatic fluid, and therefore increase the rate of chromatin fragmentation (Sailer et al., 1997). According to Sakkas and Alvarez (2010), apoptosis during spermatogenesis sometimes fails and allows some abnormal spermatogonia (with damaged DNA) to enter the maturation process. Our observations of mature spermatozoa with fragmented DNA in control group could be explained by such failures during spermatogenesis at pupal stage. The increase in DNA degradation in heat stressed group could accentuate the effect of decreased sperm vitality especially in sensitive species. As bumblebee males emerge with a fixed amount of sperm (spermatogonia have finished their maturation at pupal stage), we can reasonably exclude epigenetic alterations to gene expression following heatwave conditions (Sharma et al., 2015; Youngson & Whitelaw, 2008) as methylation (Siklenka et al., 2015) to explain DNA degradation. However, alterations in chromatin condensation (e.g. denaturation of protamines, decrease in protamine disulfide) and simple or double DNA-strand breaks induced by heat shock could explain the

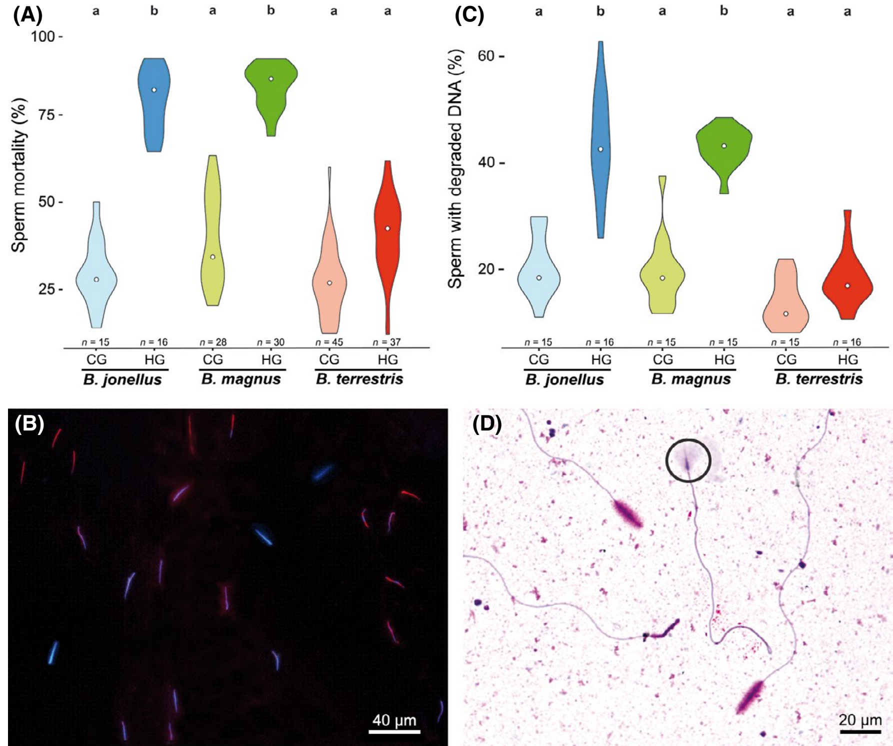

FIGURE 2 Impact of simulated heatwaves on fertility of bumblebees. (A) Violin plots of sperm mortality (%) in the three model species *Bombus jonellus (n = 31), B. magnus (n = 58)* and *B. terrestris (n = 82).* White dots represent the median for the group. Letters above the violin plots represent groups supported statistically. (B) Representative fluorescent microscopic view of spermatozoa of *B. terrestris* (from HG) treated with FluoVit kit (Microptic). Dead sperms are coloured in red (propidium iodide) while living ones are coloured in blue (DAPI). (C) Violin plots showing the percentage of spermatozoa with degraded DNA in *B. jonellus (n = 31), B. magnus (n = 30)* and *B. terrestris (n = 31).* White dots represent the median for the group. Letters above the violin plots represent groups supported statistically. (D) Representative photograph of spermatozoa of *B. jonellus* after the Sperm Chromatin Dispersion test (SCD). Four spermatozoa are shown: two with a complete halo which corresponds to spermatozoa with non-fragmented DNA, one (see dark circle) with a degraded halo indicating a fragmented sperm DNA and one without halo. CG: control group, HG: heat stressed group. For each specimen, the average of the different replicates was considered to produce the figure

observed results (Hamilton et al., 2018; Love & Kenney, 1999). The alteration of protamines due to an increase in temperature can lead to a significant decrease of fertility in mammals. Given similarities between the structure and function of protamines in humans and fruit flies (Kanippayoor et al., 2013; Rathke et al., 2007), we can suspect that a rise in internal insect temperature can have the same type of deleterious effects on protamines and also induce a significant decrease in fertilization capacity even during adult stage.

Sperm stock of bumblebees is made only once during the development of bumblebee, and it is not directly mature after bumblebee emergence (Baer, 2003). Once this stock is used by copulation, it is not renewed because testes become inactive shortly after emergence (Baer, 2003). Thus, dead sperms are not replaced by new production (Baer, 2003; Ball et al., 1983; Foitzik et al., 2002; Hölldobler & Bartz, 1985; Simmons, 2001). Moreover, in contrast to most insects (Parker, 1970), there are very few polyandrous species in bumblebees (Estoup et al., 1995; Röseler, 1973). Females of bumblebees (Chown et al., 2010; Fox & Reed, 2011) store sperm in a spermatheca after a single (i.e. monandrous, majority of bumblebee species) or multiple (i.e. polyandrous) mating for the rest of their life (Estoup et al., 1995). If a male is made less fertile by heat stress mates with a virgin queen, then it initiates a colony with a limited stock of viable sperms in queen's spermatheca (Baer, 2003). Considering that through the haplo-diploid cycle of bumblebees, a fertilized egg gives a female individual while an unfertilized egg gives a male individual (Duchateau & Marien, 1995), and modifications in the viable sperm stock could induce an increase in the ratio males/workers production and therefore a reduction in the inclusive fitness of the colony (Brown et al., 2003; Duchateau et al., 2004). Even with potential pre-copulative barriers (i.e. recognition of deviant pheromones and consecutive avoidance for more sensitive species as *B. jonellus* or *B. magnus*), negative impact can be expected as all males of the same generation are potentially exposed to heat wave during their short lifetime especially for sensitive species.

3.2 | Effect of hyperthermic shock on histology of cephalic labial glands

The cephalic labial glands (CLG) are a pair of acinar glands that represent more than half the volume of male's head. Secretions are

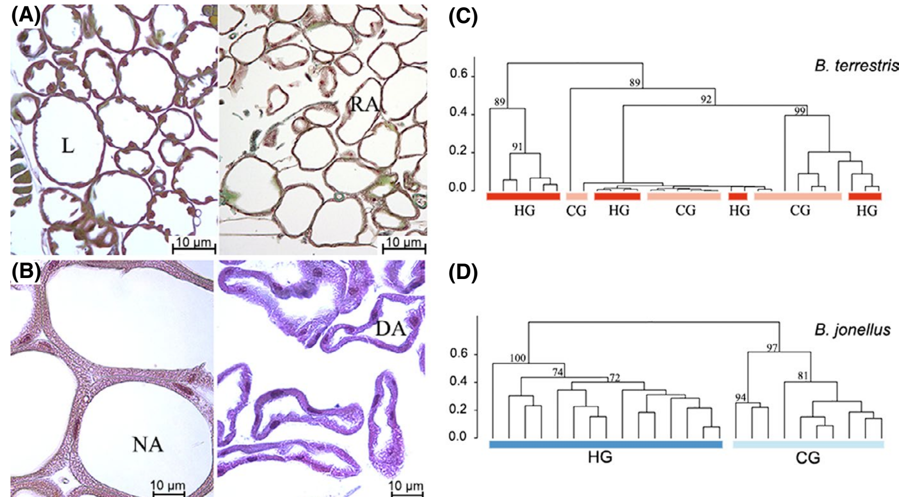

FIGURE 3 Histology of acini in the cephalic labial glands of *Bombus terrestris* (A) from control group (CG, left) and heat stressed group (HG, right) and *B. jonellus* (B) from control group (CG, left) and heat stressed group (HG, right). L = lumen of acini; RA = Acini with a restricted lumen; NA = Non-degraded (normal) acini; DA = degraded acini (heat stressed). Dendrograms based on the composition of cephalic labial gland secretions of *B. terrestris (n = 30)* (C) and *B. jonellus (n = 25)* (D). This cluster was obtained by hierarchical clustering using an unweighted pair-group method with arithmetic mean based on a correlation matrix calculated from the cephalic labial gland secretion matrix. The values near nodes represent multiscale bootstrap resampling values (only values > 70 are showed)

spilled at the base of mandibles through an excretory channel (Ågren et al., 1979). These secretions, also called pheromones, are complex mixtures composed mainly of aliphatic compounds (saturated or unsaturated hydrocarbons, esters, alcohols, aldehydes, terpenes or ketones; Ayasse & Jarau, 2014; Calam, 1969). The morphology of CLG is similar in males of *B. terrestris* and *B. jonellus* not exposed to simulated heatwave conditions (control group; Figure 3A,B). Glandular tissues are characterized by the formation of a chitinous layer above their epithelia. Acini consist of flat secretory cells whose central core is slightly flattened with abundant lipid droplets. In *B. jonellus*, lumen of acini appears to be smaller in individuals from heat stressed group than from control group ($p < 0.01$, Figure 3A,B). In *B. terrestris*, the structural shape of acini varies among specimens of heat stressed group. We observed (a) acini with a large lumen as in control group, indicating a normal production of secretions (>80%); (b) acini with a slightly reduced lumen with lipid droplets associated with the secretory cells (<15%) and (c) acini with a collapsed lumen (<5%). However, these differences between control and heat stressed groups are not supported statistically in *B. terrestris* ($p = 0.21$). The proportion of acini with a collapsed lumen (~45% of observed acini) is much higher for *B. jonellus* than for *B. terrestris*, indicating that the CLG histological structure of sensitive species (i.e. *B. jonellus*) is more impacted by heat shock ($p < 0.01$). The structure of some acini observed in stressed *B. jonellus* are similar to those observed in *B. mesomelas* where the system of sexual recognition is not based on the synthesis of cephalic labial secretions (i.e. non-functional glands; Sauter & Brown, 2001; Terzo et al., 2007). Alternatively, the structure of CLG may differ depending on species (i.e. specific functional structure) and age of specimens (Sobotnik et al., 2007; Terzo et al., 2007; Žáček et al., 2013). The maturity of CLG is reached after 7 days of emergence in *B. lucorum* and *B. terrestris* (Žáček et al., 2013), related to a peak activity of the gland. Some parts of the gland of *B. jonellus* (heat stressed group) could also correspond to inactivated acini with a reduced production of secretions after exposure to hyperthermic shock. Analyses of secretion composition (see below) seem to indicate that glands are impacted but still partly functional.

3.3 | Effect of hyperthermic shock on cephalic labial gland secretions

We detected 102 compounds in *B. terrestris* and 45 compounds in *B. jonellus* (see Table S2). Regarding the sensitive species *B. jonellus*, cluster analysis shows a clear differentiation between control and heat stressed groups (Figure 3C,D). This separation based on heat exposition is supported by high bootstrap values and perMANOVA test ($p < 0.01$). Indval's analysis revealed 10 indicator compounds for which the abundance was significantly different between control and heat stressed groups in *B. jonellus* (e.g. dihydrofarnesyl dodecanoate, nonacos-9-ene, nonacos-7-ene, dihydrofarnesyl tetradecanoate, dihydrofarnesyl octadecenoate, geranylgeranyl decanoate). Regarding the resistant species *B. terrestris*, even for minor compounds, our chemical analyses revealed no significant changes between control and heat stressed groups ($p = 0.73$).

Slight quantitative modifications in pheromone mixtures may impede nuptial behaviours and consequently the conspecific recognition of sexual partners (Bergström et al., 1981; Boullis et al., 2016; Dobzhansky & Gould, 1982; Lecocq et al., 2015). Females of *B. terrestris* do not react only to the perception of major compounds (Terzo et al., 2007). Minor changes in pre-copulatory behaviour could also affect potential selection of males (Sobotnik et al., 2007). However, our results show that, contrary

to cold-adapted species, heat shock has a limited impact on the attractiveness of males of *B. terrestris* (i.e. a warm-adapted species), which suggest that virgin queens of *B. terrestris* would be unable to distinguish secretions of heat stressed individuals from secretions of non-stressed individuals. It seems there is no pre-copulative barrier (i.e. CLGS) to avoid copulation between virgin queens and males exposed to hyperthermic shock. If female preference for these secretions predicts mate choice, hyperthermic shock may not have an impact on population dynamics especially if sperm quality is not affected as demonstrated in this study. A previous study in *Nasonia vitripennis* (a parasitic wasp) also showed that females are not able to distinguish a heat stressed male from a non-stressed male (Chirault et al., 2015). However, CLGS are not the unique pre-copulative barrier in bumblebees; there are also visual signals (Ayasse et al., 2001) involved in nuptial behaviour. Moreover, copulation tests are needed to investigate if stressed males are able (through neuromuscular potential, water balance or physiological disruptions) to mate with virgin queens with or without potential dramatic consequences on the offspring.

3.4 | Species specificity of heat shock damages

Heat stress impacts bumblebee populations through direct (i.e. mortality, drastic reduction of populations; Martinet et al., 2015; Rasmont & Iserbyt, 2012) or indirect effects (i.e. decrease in fertility as shown in the present study). Previous studies showed that bumblebee species are not equally affected by heat stress (Martinet et al., 2015; Oyen et al., 2016). Depending on species traits (increasing and heat-resistant vs. declining and heat-sensitive), our results suggest that bumblebee spermatozoa have a species-specific sensitivity to hyperthermic shock. This could have a critical effect on bumblebee fertility and fitness. These results on bumblebees are in the line of previous studies on fertility in zebrafish *Danio rerio*, fruit flies *Drosophila*, beetles *Tribolium castaneum* and bees *Apis mellifera* where sperm stock is reduced with potential sperm DNA damages after heat exposure (David et al., 2005; Hurley et al., 2018; Stürup et al., 2013). Moreover, in diverse organisms, several authors have shown that males are more impacted by hyperthermic conditions than females (e.g. David et al., 2005; Hurley et al., 2018; Janowitz & Fischer, 2011; Setchell, 2006). However, while males can die in heat conditions that are tolerated by females, the viability of stored sperm in spermatheca decreases with heat exposure (McAfee et al., 2020; Pettis et al., 2016). These reproductive traits should be therefore included in the framework of risk assessment to explain pollinator decline or resilience.

The limited effect of heat shock on fertility and attractiveness of *B. terrestris* could partly explain its invasiveness. Several studies have shown that *B. terrestris* is a ubiquitous, polylectic and resistant species to environmental stresses such as parasites, non-adequate diet or thermic treatment (Graystock et al., 2016; Rasmont et al., 2015; Vanderplanck et al., 2019). Moreover, this species is invasive in some regions such as Japan or South America (Inari et al., 2005; Torretta et al., 2006) and displays a considerable flexibility in the seasonal timing of colony development (e.g. summer aestivation or multi-voltinism; Jones et al., 2004; Rasmont et al., 2008). Although *B. terrestris* could be described as a thermo-tolerant species, lack of effects induced by extreme temperatures could reflect a difference of temperature optimum (operating range) compared to more cold-adapted species such as *B. magnus* or *B. jonellus*. Therefore, a heatwave temperature of 40°C may not represent a significant biological stress for *B. terrestris* in contrast to sensitive species which are strongly impacted by such temperatures. However, in addition to being a heat-tolerant species, *B. terrestris* displays a minimum critical temperature (CT_{min}) which range from −5°C to −9°C (Owen et al., 2013) while Pimsler et al. (2020) have measured a CT_{min} between 0°C and −6°C for the North American bumblebees, *B. vosnesenskii*. Taking this into account, *B. terrestris* seems to be a species able to tolerate a large range of low and high temperatures compared to other bumblebee species.

4 | CONCLUDING REMARKS

This study sheds light on the assessment of reproductive fitness of survivors after hyperthermic shock and shows that extreme climatic events could have species-specific effects on nuptial behaviour and biological reproduction of bumblebees. We have assessed the effect of simulated heat waves on key male reproductive traits leading to a potential breakdown of mating. Our results demonstrate the resistance and survival potential of invasive and warm-adapted species (here *B. terrestris*) in comparison to spatially restricted and cold-adapted species (here *B. jonellus* and *B. magnus*). Despite difficulties of sampling and breeding for sensitive species such as *B. magnus* and *B. jonellus*, further analyses of attractive bioassays are necessary to explore consequences of cephalic labial gland degradation after heat exposure observed in this study on attractiveness with virgin queens of sensitive species. For more sensitive species, potential effects of heat shock on female perception of male pheromones during nuptial courtship (patrolling behaviour) could have a severe impact on bumblebee populations by preventing meeting of sexual partners. In addition, this risk should be increased if sperm DNA is degraded or if mortality exceeds the minimum threshold for ensuring optimal fertilization of eggs. Further experiments on copulation, sperm competitiveness, quality of sperm stored in spermatheca, offspring development produced with males exposed to heatwave conditions (transgenerational damage) and adult life span of offspring are necessary to assess complete damage of heatwaves. Our results also suggest that all species are not equal when exposed to hyperthermic stress potentially induced by heatwaves. We highlight the value of monitoring and evaluating a wide range of species to provide a comprehensive view of different threats for pollinators. This framework could ultimately serve as a basis for discussing practical solutions for wild populations in order to safeguard one of the most important actors for ecosystem services.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

Conception and design of the experiments: B.M., E.Z., K.P., A.A., D.N., P.R., E.H.; Experimentation: B.M., E.Z., K.P.; Data analysis: B.M., E.Z., K.P., D.N., E.H.; Paper writing: B.M., E.Z., K.P., T.L., D.N., P.R., D.M., E.H.

DATA AVAILABILITY STATEMENT

All data are available in the main text or in the Supporting Information. Full data sets with the global sampling are available in Supporting Information (electronic-only appendices) but also from the Dryad Digital Repository https://doi.org/10.5061/dryad.mkkwh70z1 (Martinet et al., 2020).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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